THE BIOCHEMICAL JOURNAL

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Aspects of the Metabolism of Glycine and of Porphyrins

BY ALBERT NEUBERGER

THE SECOND HOPKINS MEMORIAL LECTURE

Delivered to a meeting of The Biochemical Society on 29 June 1960 in the Anatomy Lecture Theatre, Cambridge University

An invitation to give a Hopkins Memorial Lecture is the greatest honour which The Biochemical Society can confer upon one of its members and I wish to thank you for awarding me this distinction which I deeply appreciate. The pleasure which such an invitation must necessarily produce in the recipient is mingled, however, with a feeling of humility and inadequacy to perform such a task. Unlike my distinguished predecessor, Sir Rudolph Peters, I was associated with Sir Frederick Gowland Hopkins only for the comparatively short period of three-and-a-half years, and this occurred at a time when my scientific interests had already been defined to a large extent by having worked for six years in the department of Sir Charles (then Professor) Harington. During the time I spent in the Biochemistry Department in Cambridge I was in fairly close and frequent contact with Hopkins and I am conscious of having been deeply influenced by the scientific philosophy and the teachings of that great master. The important part played by Hopkins in the development of British and indeed world biochemistry has been described by others who are better qualified than I am to evaluate his contribution to biological sciences. In choosing as a subject of this lecture the metabolism of glycine, serine and porphyrins, I was actuated by two considerations. It is a field to the knowledge of which my colleagues, past and present, and myself have made some contributions; but this choice also gives me the opportunity to demonstrate the wide sweep of biochemistry covering the whole of living matter from microbes to man, and to show the variety of methods employed, which range from investigations in the whole animal to experiments with a pure enzyme and which include in my opinion most of the techniques used by the organic chemist.

Glycine was first isolated in 1820 by Braconnot from a hydrolysate of gelatin and was first synthesized by Perkin and Duppa in 1858. It is present in all or almost all proteins so far analysed, but, apart from special groups of proteins, its concentration in terms of grams of amino acid/100 g. of protein hardly ever exceeds 6. The exceptions are the keratins, in which glycine contents vary between 5 and 9·5%, the mammalian collagens, with 26–27% of glycine, and fibroins, which contain between 23 and 44% of glycine. As collagen forms about 20–25% of the total protein of the mammal, and as this protein contains about 26% of glycine, it follows that approximately 60% of the glycine of the organism is bound in collagen linkage. Since the latter is metabolically fairly inert (Neuberger, Perrone & Slack, 1951), it appears that more than half of the glycine is in a state in which it does not take a quantitatively significant part in the relatively fast synthetic and degradative processes in which most other body constituents continuously participate.

It has been known for some time that glycine can be synthesized by mammals, and Rose and his co-workers have shown that the presence of glycine in the diet of the rat is not required for maximum growth; the same is likely to apply to man and probably most other mammals. In the young chick and turkey, growth rate is reduced if glycine is completely absent from the diet (Almquist, Stokstad, Mecchi & Manning, 1940), but glycine can be replaced by serine or aminoethanol.

Table 1. *Biological reactions involving glycine*

Incorporation into proteins
Conversion into carbohydrate
Formation of creatine
Incorporation into purines
Formation of serine
Formation of glutathione
Formation of glycocholic acid
Formation of hippuric acid
Oxidation or transamination to glyoxylic acid
Formation of aminolaevulic acid and aminoacetone
Reversible transformation into *allo*threonine (and threonine)

Table 2. *Biological reactions of serine*

Incorporation into proteins
Splitting into glycine and 'formaldehyde' (production of C_1 units from β-carbon atom)
Deamination to pyruvate
Oxidation or transamination to hydroxypyruvate
Reaction with homocysteine to cystathionine
Decarboxylation to aminoethanol
Incorporation into phospholipid
Reaction with palmitaldehyde to dihydrosphingosine
Condensation with indole to tryptophan

For the adult bird glycine is not required, not even for egg-laying (Johnson & Fisher, 1956). The reasons for the special needs for glycine by the young bird are considered below.

Glycine takes part in a great number of biochemical reactions, as the representative, but incomplete, list (Table 1) shows. It is now well established that glycine and serine are readily transformed into one another, and therefore in an overall assessment of the biochemical importance of glycine it is necessary to take into account the various reactions in which serine participates (Table 2). It would be impossible in the course of this lecture to deal adequately with all the various transformations involving glycine and serine which occur in the different forms of life. I have therefore chosen a few topics which for various reasons seem most appropriate for this occasion.

The enzymic condensation of glycine with acyl-coenzyme A derivatives

The reactions leading from glycine to porphyrins have now in general been well established, mainly owing to the work of the Laboratories of Shemin, Rimington and those of my own group, and the later stages in this sequence of enzymic steps are now being elucidated in the schools just mentioned and in the Laboratories of Granick and Bogorad. In this lecture I wish to confine my attention to the earliest reaction, i.e. the condensation between glycine and succinyl-coenzyme A (CoA).

In the reaction between glycine and acyl derivatives of coenzyme A to yield α-aminoketones and carbon dioxide, pyridoxal 5-phosphate is required as a cofactor (Laver, Neuberger & Udenfriend, 1958; Gibson, Laver & Neuberger, 1958; Kikuchi, Kumar, Talmage & Shemin, 1958; Kikuchi, Kumar & Shemin, 1959), and it is reasonable to assume that the actual reactant is a Schiff's base or aldimine formed between the amino group of glycine and the carbonyl group of pyridoxal phosphate. As pointed out by Lynen (1959), acyl-CoA compounds can act either as nucleophilic or as electrophilic reagents and in δ-aminolaevulic acid formation it is likely that the acyl carbon atom of succinyl-CoA functions as electron acceptor to the nucleophilic reagent, which in this case is the anion formed by the removal of a proton from the α-carbon atom of the glycine–pyridoxal phosphate complex. By following largely the suggestions of Braunstein and Snell (see also Metzler, 1957), the initial reaction may be visualized as in Fig. 1.

It is assumed that the proton which dissociates from the α-carbon atom of the glycine moiety migrates to the ring nitrogen atom, the resulting zwitterionic structure (I) being stabilized by resonance with a non-zwitterionic form (II) in which all double bonds including that of the carboxylate group are conjugated. This resonance facilitates dissociation and migration of the proton and the carbanion formation is further assisted, as suggested by Snell, by hydrogen-bond formation between the nitrogen atom derived from glycine and the phenolic oxygen atom. It is further proposed that the complex is linked to protein, probably by ionic forces, and that such linkage

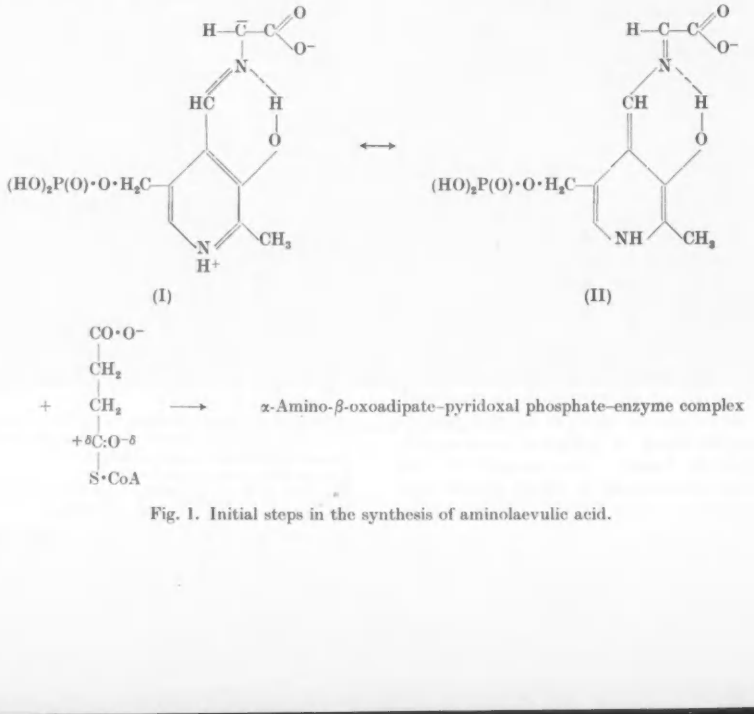

Fig. 1. Initial steps in the synthesis of aminolaevulic acid.

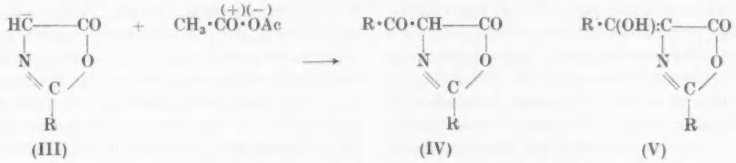

Fig. 2. Mechanism of the formation of acylaminoketones.

takes place through the phosphate and carboxylate groups. A linkage through the carboxylate group to protein has been suggested for amino acid decarboxylases by Rothberg & Steinberg (1957), and is likely to consist of a Coulomb type of interaction with a positively charged group on the enzyme. The masking of the negative charge of the carboxylate group will also assist the ionization of the proton in the α-position, i.e. the formation of the postulated carbanion. The aminoketone synthetase appears to be fairly non-specific with respect to the acyl group of the acyl-CoA derivative, but the only amino acid which has so far been found to give an aminoketone with the enzyme system is glycine. This specificity suggests that glycine is directly attached to the enzyme protein. With most purified enzymes of animal origin requiring pyridoxal phosphate so far examined, it has been found that the cofactor is fairly strongly bound to the protein. This general finding is supported by observations made on the aminoketone synthetase obtained from bird erythrocytes. Such preparations have, even after prolonged dialysis, considerable activity without addition of pyridoxal phosphate. However, addition of the coenzyme produces a further increase of activity which is not reduced by further dialysis. Enzyme preparations from microbial sources such as *Rhodopseudomonas spheroides* behave differently and even a concentrated extract of bacterial cells shows no activity unless pyridoxal phosphate is added. Another relevant observation is that the inhibition which is caused by cysteine and penicillamine is stereospecific. As suggested by du Vigneaud and his colleagues, this inhibition is almost certainly due to a formation of a thiazolidine ring with participation of the aldehyde group of pyridoxal phosphate. As pyridoxal phosphate itself and glycine are optically inactive, the very specific inhibitory power shown by the L-enantiomorphs of these α-amino β-thiol acids can most readily be explained by the assumption that the coenzyme is combined with an optically active substance which most likely is the enzyme.

Almost all reactions promoted by enzymes can be carried out by the organic chemist with more vigorous conditions or unphysiological catalysts. The enzymic formation of α-aminoketones from glycine and activated derivatives of carboxylic acids has an apparent parallel in the reaction described by Dakin & West (1928), in which α-acetamidoketones are produced by warming an amino acid with acetic anhydride in the presence of pyridine. Indeed Wiley (1950) has suggested that the α-acetamido acid which is first produced is converted into a carbanion, with simultaneous loss of carbon dioxide, and that this ion then reacts with acetic anhydride to give the acetamidoketone. However, Cornforth & Elliott (1950) have put forward fairly convincing arguments that the more likely reactant is the ion derived from a 2-methyl-5-oxazolone (III), which on reaction with the acid anhydride will give a 4-acyloxazolone (IV or V; R = CH_3) (Fig. 2); the latter, on ring-opening, which occurs readily, will lose carbon dioxide and thus give an acylaminoketone. We ourselves have found that α-acetamido β-oxo acids are fairly unstable. The recent experiments of Crawhall & Watts (1960), however, suggest that the mechanism suggested for acetylative decarboxylation by Cornforth & Elliott may either have to be modified or that there may be a second mechanism.

The close resemblance between the oxazolone structure and the intermediate proposed in the aminoketone-synthetase reaction (structures I↔II) is obvious and in both cases the carbon–nitrogen double bond assists the adjacent carbon atom to lose a proton or to react as a nucleophilic centre. Another closely related example is the reaction used by Attenburrow, Elliott & Penny (1948) in their synthesis of threonine. These workers obtained by condensation of acetic anhydride with hippurate in the presence of base a substance which is in fact the azlactone (IV) or (V) (R = C_6H_5) of α-benzamido-β-oxobutyric acid. It thus appears that the enzymic reaction under consideration is based on the reactivities of the various groups which can be ascertained by studying the behaviour of the compounds in ordinary organic reactions.

The decarboxylation step

When δ-aminolaevulic acid was discovered to be an intermediate in porphyrin synthesis, both Dr Shemin's group and we assumed that α-amino-β-oxoadipic acid was the first product of the enzymic

reaction and that this compound then lost carbon dioxide either spontaneously or by the action of a decarboxylase. As such compounds had not been synthesized, it seemed desirable to obtain some information about their properties or indeed to ascertain whether they have even a transient existence. It was aimed (Laver, Neuberger & Scott, 1959*a*, *b*) to obtain the free α-amino β-oxo acid by a synthesis, the last step of which consisted in the liberation of the sensitive carboxy group by the mildest possible means. The various reactions are shown in Fig. 3. Ethyl β-oxoadipate was treated with benzyl alcohol under conditions which led to an ester exchange on the carboxyl group which is in β-position to the keto group. The mixed ester (VI) was then nitrosated and the oximino keto acid ester (VII) was reduced in acid solution; the acidity was such that only the ethyl ester group was hydrolysed. The difference in the rate of splitting of the two ester groups is caused by the fact that the positive charge of the amino group repels the approach of the proton or hydronium ion to the α-ester grouping to a much greater extent than it does to the more distant δ-ester grouping. The α-benzyl ester of α-amino β-oxoadipic acid (VIII) could thus be obtained in good yield. The free acid was formed by catalytic reduction of the benzyl ester with the use of a large amount of catalyst, but on isolation only the decarboxylation product, aminolaevulic acid (IX), was obtained. By measuring the volume changes during catalytic reduction and changes in ultraviolet absorption, it appeared that the maximum half-life of the dicarboxylic acid is about 20 min. in ethanol and approximately 8 min. in water at pH 0. At pH 7·0 the half-life is less than 1 min. and it is clear that the likelihood of demonstrating even the transient existence of this aminoketodicarboxylic acid in a biological system is at present rather remote.

The observations described render the assumption of a participation of an enzyme in the decarboxylation of aminolaevulic acid unnecessary. Moreover, the structures postulated above (I ↔ II) for the intermediate in the condensation of glycine with succinyl-CoA, i.e. the complex of pyridoxal phosphate, glycine and the enzyme, are identical with those proposed to be concerned in the enzymic decarboxylation of many amino acids not containing a β-carbonyl function. It would thus be reasonable to assume that condensation of the aldimine with succinyl-CoA is followed by a second reaction consisting in the decarboxylation of the pyridoxal phosphate–α-amino-oxoadipic acid complex or that indeed both reactions are simultaneous. It would seem difficult to devise experiments which could decide between these two possibilities, i.e. spontaneous non-enzymic decarboxylation and participation of a pyridoxal phosphate-containing enzyme, and we were prepared to leave this question open, had it not been for other developments in this field which I now wish to relate.

Effect of biotin on porphyrin formation and on synthesis of δ-aminolaevulic acid

It was shown by Lascelles (1956) that suspensions of the photosynthetic organism *Rhodopseudomonas spheroides* grown in the light and in a medium deficient in biotin do not form coproporphyrin or bacteriochlorophyll from α-oxoglutarate and glycine unless further incubation is carried out in the presence of biotin. The last-named is not required for porphyrin synthesis from aminolaevulic acid. My colleagues K. D. Gibson and G. H. Tait have extended this work further. They have shown that biotin is also required for the synthesis of aminolaevulic acid from glycine and succinate, and the latter is presumably transformed into succinyl-CoA, not by the α-oxoglutaric acid dehydrogenase, but by the enzyme succinyl thiokinase. It seemed therefore possible that biotin is needed for the actual condensation of succinyl-CoA and glycine. It was further found that deficiency of biotin in the medium reduces not only porphyrin formation in whole cells, but also the level of aminolaevulic acid-synthetase activity in cell extracts, and this decrease is very much greater than that of aminolaevulic acid-dehydrase activity. Whilst the decrease of porphyrin pro-

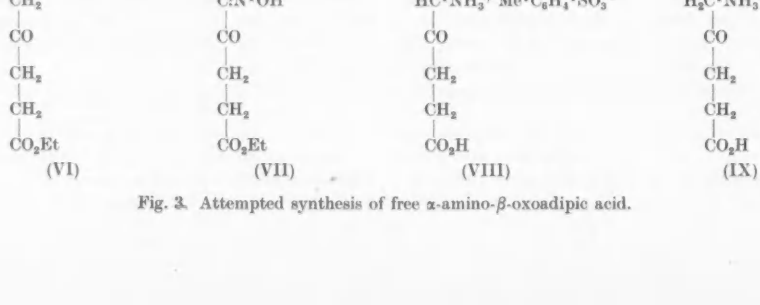

Fig. 3. Attempted synthesis of free α-amino-β-oxoadipic acid.

duction in biotin-deficient cells may be caused by a possible participation of biotin in the synthesis of pyridoxal phosphate, or some other reaction not involving the actual synthesis of aminolaevulic acid, the apparently specific reduction of aminolaevulic acid synthesis in extracts in which succinyl-CoA is used as substrate in the presence of added pyridoxal phosphate clearly indicates that biotin is either a coenzyme in the actual condensation or participates specifically in the synthesis of the protein without being incorporated into its structure.

Mr Tait has recently shown that addition of a commercial preparation of avidin to a cell extract inhibits synthesis of aminolaevulic acid from succinyl-CoA and glycine. Avidin is a protein occurring in egg white and known to bind biotin strongly and, so far as is known at present, specifically. This observation was confirmed by using a number of pure proteins isolated from egg white by Dr N. M. Green and Dr R. D. Marshall. Of all the fractions used, inhibition was found only with an avidin fraction which was at least 50% pure. So far it has not been possible to overcome the inhibitory effect of avidin by prior incubation with biotin, and this must necessarily lead to some doubt as to whether the reduction of the synthesis of aminolaevulic acid by avidin is caused by an interaction with biotin. It is of course not impossible that enzyme-bound biotin displaces free biotin from the complex, and this and other possibilities are being explored at present. We cannot assume that the belief which is generally held at present that avidin interacts only with compounds of the biotin type is securely established. On the other hand, the facts that biotin deficiency results in an apparently specific reduction in aminolaevulic acid-synthetase activity in cell extracts and that aminolaevulic acid synthesis is inhibited by avidin may be considered as indications that biotin may play an integral part in the synthesis of aminolaevulic acid by the synthetase. Lynen (1959) has shown that free biotin combines with carbon dioxide through one of the ureido nitrogen atoms, forming an allophanic acid type of structure. If biotin functions directly in the synthesis of aminolaevulic acid we may picture the formation of the acid as being a concerted reaction in which the condensation of the enzyme–glycine–pyridoxal phosphate complex with succinyl-CoA is synchronized with the removal of carbon dioxide as a biotin derivative. My colleague Dr J. J. Scott has suggested that the complex formed between α-amino-β-oxoadipic acid, pyridoxal phosphate and the enzyme may be very stable and the intervention of protein-bound biotin is needed to remove the carboxyl group, and this results in the regeneration of the enzyme and pyridoxal phosphate together with the formation of aminolaevulic acid. But it should be emphasized that this idea is at present only a somewhat adventurous speculation and that further work is needed with a highly purified enzyme before direct participation of biotin in the actual synthesis of aminolaevulic acid can be considered established.

In addition to the decrease in the activity of the aminolaevulic acid synthetase which can be measured in cell extracts by the addition of succinyl-CoA and glycine and pyridoxal phosphate and which is observed in severe biotin deficiency, there is another effect of biotin on porphyrin synthesis which must be more indirect. If the concentration of biotin in the medium is about 2 $\mu\mu$M, aminolaevulic acid-synthetase activity in cell extracts is almost normal, but no porphyrin or bacteriochlorophyll formation is found, in spite of the presence of apparently adequate amounts of enzyme. However, such cells can be induced to produce normal amounts of porphyrin if ethionine is added to the medium at a concentration of 10^{-3}–10^{-4} M. Porphyrin formation can also be inhibited by a marked increase of the glycine concentration in the medium, and this decrease can be prevented if, together with an excessive amount of glycine, ethionine is added to the medium. We are not at present able to suggest a really satisfactory explanation for these findings, but it seems likely that aminolaevulic acid synthesis in the cell is regulated by as yet unknown factors in addition to that of the availability of aminolaevulic acid synthetase. Such factors may be the formation of pyridoxal phosphate, that of succinyl-CoA, the rate of competing reactions or the penetration of glycine into the cell. Biotin may be involved in one or other of these reactions, and the ethionine effect may be caused by an interference with the formation of an inhibitor or by interference with a reaction which competes with the synthesis of aminolaevulic acid or with its transformation into porphobilinogen.

The possibility occurred to us some time ago that biotin may be required, not by acting as a coenzyme in the actual condensation, but by preliminary activation of glycine through carboxylation to aminomalonic acid. If this were the case, the aminomalonate ion with its high reactivity on the α-carbon atom would function in a manner similar to that of malonyl-CoA in acetoacetate formation. However, Miss M. Matthew in my Laboratory has found that aminomalonate was not a substrate in the enzymic condensation with succinyl-CoA. Indeed it proved to be a powerful inhibitor. With a ratio of glycine to aminomalonate of 80:1, inhibition amounted to 20%, whilst with a ratio of 15:1 the inhibition was almost complete.

General metabolic importance of aminoketone formation

I have referred to the fact that the enzyme aminolaevulic acid synthetase is not very specific with regard to the acyl group of the coenzyme A derivative, and all aliphatic mono- and di-carboxylic acids which have been examined give rise to aminoketones under the catalytic influence of the rather impure aminolaevulic acid-synthetase preparations available. As acetyl-CoA and malonyl-CoA are formed in relatively large amounts in the body, we should expect aminoacetone and γ-aminoacetoacetic acid to be synthesized by cells and to be found in Nature. The formation of aminoacetone by *Staphylococcus aureus* has indeed been demonstrated by Elliott (1958, 1960), and Mr Tait and I have recently shown that *Rhodopseudomonas spheroides* can form an aminoketone which was different from aminolaevulic acid and could be identified as aminoacetone after incubation under certain conditions. Aminoacetone has also been identified as a hydrolysis product of the antibiotic micrococcin P by Mijović & Walker (1960), and the occurrence in urine of a compound having the properties of aminoacetone has also been reported (Mauzerall & Granick, 1956). γ-Aminoacetoacetic acid has not yet been shown to occur in Nature, but it has been synthesized by Neilands, Neuberger & Scott (1959). Interestingly enough, this γ-amino keto acid is much more stable than α-amino-β-oxobutyric acid, and on decarboxylation gives rise to aminoacetone, suggesting a second pathway of its biological formation. The γ-amino-β-oxobutyric acid, if it is formed biologically, might also produce, by reduction of the keto group, γ-amino-β-hydroxybutyric acid, which is presumably the precursor of the betaine carnitine, a substance present in muscle in relatively large amounts and also a growth factor for insects. Finally, there is the possibility that the D-1-aminopropan-2-ol moiety in vitamin B_{12}, which Sprinson and his colleagues (Krasna, Rosenblum & Sprinson, 1957) have shown to be derived biologically from L-threonine, is formed through aminoacetone by reduction. Tait and I have recently obtained an enzyme from *Rhodopseudomonas spheroides* which oxidizes L-threonine to aminoacetone with decarboxylation. This enzyme uses DPN^+ (diphosphopyridine nucleotide) specifically, no activity being observed if TPN^+ (triphosphopyridine nucleotide) is employed. The enzyme acts on L-threonine and on D-*allo*threonine, the two isomers with D-configuration on the β-carbon atom. D-Threonine and L-*allo*threonine are not attacked.

This particular enzyme does not act on DL-serine, but the possibility exists that other aminoketones may be formed by a pathway other than the condensation of glycine with acyl-CoA derivatives. Thus succinic semialdehyde may be condensed with glycine–pyridoxal phosphate to give α-amino-β-hydroxyadipic acid, which could be oxidized to aminolaevulic acid by an enzyme similar to that oxidizing threonine to aminoacetone.

Shemin, in his first paper on the role played by aminolaevulic acid in porphyrin formation, suggested that this amino keto acid may play a part in the general metabolism of glycine. Shemin and his colleagues (Shemin, 1955; Kikuchi, Kumar & Shemin, 1959; Nemeth, Russell & Shemin, 1957) have extended their scheme, which was originally named the succinate–glycine cycle, from aminolaevulic acid to aminoacetone, and they have produced evidence showing that the carbon atoms carrying the amino group in the two aminoketones are precursors of C-2 and C-8 of guanine and of uric acid and also of formate. Elliott (1960) has described an enzyme from ox plasma which converts aminoacetone into methylglyoxal, and the Columbia group has shown that $\gamma\delta$-dioxovaleric acid ('ketoglutaric semialdehyde') produces a similar pattern of labelling of uric acid as does aminolaevulic acid. The actual enzymic steps involved have not yet fully been elucidated, and, whilst good evidence exists for the occurrence of the reactions postulated, the quantitative significance of this reaction for the overall metabolism of glycine remains still to be established.

The glycine–serine transformation

It was shown by Shemin (1946) that serine is a very effective precursor of glycine in the whole rat, and Sakami (1948) produced evidence for the occurrence of the inverse reaction. The enzymic mechanisms involved have been elucidated by Blakley, who started his work in this field in my laboratory in Mill Hill and continued it in Canberra (see, for example, Blakley, 1955), by Kisliuk & Sakami (1954), by Huennekens, Whiteley & Osborn (1959), and it is now possible to give a complete description of the sequence of reactions concerned. Folic acid, or rather tetrahydrofolic acid, and pyridoxal phosphate have been shown to be required and the C_1 unit involved is of the oxidation state of formaldehyde. Starting with formaldehyde and glycine, the reaction can be represented by the following equations:

(1) H·CHO + tetrahydrofolic acid $\rightleftharpoons$ 'hydroxymethyl'-tetrahydrofolic acid

(2) 'Hydroxymethyl'-tetrahydrofolic acid + glycine–pyridoxal phosphate $\rightleftharpoons$ serine + pyridoxal phosphate + tetrahydrofolic acid.

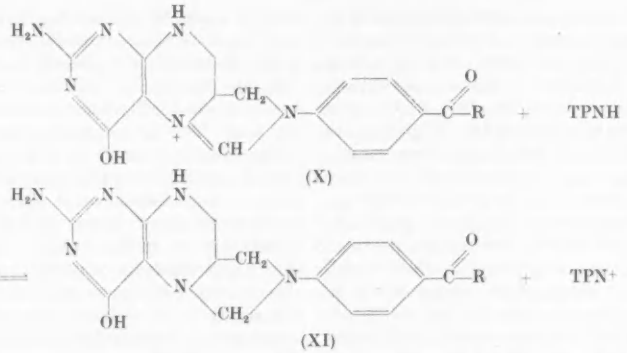

Fig. 4. Reversible oxidation and reduction involving methinetetrahydrofolic acid.

Probable structures of the amino acid–pyridoxal phosphate complexes have been proposed by Snell and his co-workers and also by Braunstein, and are very similar to those discussed above in connexion with the aminolaevulic acid-synthetase reaction. For the 'active formaldehyde' Blakley and I suggested a structure in which a methylene group joins N-5 and N-10 in tetrahydrofolic acid to form a new ring (Fig. 4; XI). N^5N^{10}-Methylenetetrahydrofolic acid can be oxidized to N^5N^{10}-methinetetrahydrofolic acid (X) with the aid of a TPN-requiring dehydrogenase and thus give rise to N^{10}-formyltetrahydrofolic acid and further to the C-2 and C-8 atoms in purines (for review see Huennekens *et al.* 1959). The C_1 unit can also be reduced to a methyl group reacting with adenosylhomocysteine to give methionine or yield the methyl group in thymine. The first reaction mentioned above, the condensation of formaldehyde with tetrahydrofolic acid, occurs at a reasonable rate on just mixing the two reactants, but Osborn, Vercamer, Talbert & Huennekens (1957) have shown that pigeon liver contains an enzyme which catalyses this reaction.

On the basis of these enzymic reactions it is easy to see how the organism can synthesize glycine from serine, if the latter can be made from substances involved in either the tricarboxylic acid cycle or glycolysis. But it appears that the reverse reaction, the formation of serine from glycine, also occurs readily and the origin of the formaldehyde required cannot be indicated with certainty. There are of course present in the body a number of compounds containing active methyl groups, such as methionine and choline, but these are not made or are not present in large amounts in the body, and the same applies to substances such as histidine or tryptophan, whose catabolism gives rise to formate. Acetone has been shown to be a precursor of C_1 units, but it is doubtful whether this can represent a readily available store of 'active' formaldehyde. It must be pointed out that not all the radioactivity found in serine on administration of labelled glycine to an animal may represent a net synthesis, and at least part of it may result from an exchange reaction:

$$H_2N\cdot\overset{*}{C}H_2\cdot CO_2H + HO\cdot CH_2\cdot CH(NH_2)\cdot CO_2H \rightleftharpoons H_2N\cdot CH_2\cdot CO_2H + HO\cdot CH_2\cdot\overset{*}{C}H(NH_2)\cdot CO_2H.$$

(* indicates a labelled carbon atom)

Secondly, the α-carbon atom of glycine itself may be a precursor of C_1 units. Sakami (1949) showed that, on administration of large amounts of α-labelled glycine, serine could be isolated which contained the label in almost equal amounts in the α- and β-positions of the molecule, indicating that the α-carbon atom of glycine can give rise to a C_1 fragment. Arnstein & Neuberger (1953*b*) showed that this labelling pattern was observed only if the glycine content of the diet was 2%; with 0·5% of glycine, the specific radioactivity of the β-carbon atom of serine was only 20% of that of the α-carbon atom. However, it appears that glycine may make, under conditions of normal nutrition, a significant contribution to the supply of C_1 units in the body. There are two known metabolic pathways to account for the formation of C_1 units from glycine. As already discussed, the condensation of glycine with various acyl-CoA derivatives leads to δ-aminolaevulic acid and aminoacetone, both of which have been shown by Shemin and his colleagues to produce C_1 units which are ultimately derived from the α-carbon atom of glycine. It is still unknown whether the aminoketone condensation takes place on a quantitatively important scale outside the cells specifically concerned with erythropoiesis, and further work is required to assess the significance of this type of reaction for the general metabolism of glycine. The second

possibility is an oxidation of glycine to glyoxylic acid or the formation of the latter by transamination (Nakada & Weinhouse, 1953). Glyoxylic acid will give rise to formate by known pathways. Arnstein (1954) has pointed out that if glyoxylic acid were an important metabolite of glycine, it would be expected (see Weinhouse & Friedmann, 1951) that feeding of glycine would yield oxalate. But this is not the case. It seems possible, however, that this type of argument is not valid. Sanadi & Bennett (1960) have recently described a particulate enzyme system from the mitochondrial fraction of chicken liver which brings about a net synthesis of serine from glycine, and this reaction does not require the addition of a folic acid derivative or of glyoxylic acid; moreover, added formaldehyde does not participate in serine formation in this system. It is conceivable that the reactions involved are those known already, i.e. formation of glyoxylic acid, followed by decarboxylation, reduction of formate to formaldehyde and addition of the latter to a second glycine molecule to give serine. But the reactions might take place inside the organized mitochondrial structure and the intermediates would then not be in equilibrium with external glyoxylic acid formaldehyde.

Quantitative aspects of the formation of glycine and serine

Some years ago, Arnstein and I (Arnstein & Neuberger, 1953*a*, *b*) carried out some experiments in rats which were intended to give information about the rates of synthesis in the intact animal of glycine and serine, the availability of the various carbon atoms of these two amino acids for the formation of labile methyl groups and the effect of vitamin B_{12} on these processes. Dr Arnstein studied the effect of deficiencies of other vitamins on the biosynthesis of glycine (Arnstein & Stanković, 1956) and compared alanine and glucose as precursors of serine and glycine (Arnstein & Keglević, 1956). In all these experiments, known amounts of isotopically-labelled amino acid were fed as a component of a diet of known composition over long periods extending to 39 days to animals which in most cases were growing. At the end of the experiments, amino acids were obtained from the protein of the viscera after hydrolysis and the specific radioactivity of individual carbon atoms was measured after application of the appropriate chemical techniques. Comparison of the radioactivities of the fed and of the isolated amino acid and knowledge of the total amount of labelled amino acid eaten by the animal allowed an estimate to be made of the amount formed by the animal from precursors. It was assumed that at the end of the experiments all the amino acids in the protein had been replaced by amino acids derived from the diet or made by the animal, but this assumption may not have been completely correct. But a more doubtful hypothesis underlying this approach is that the fed amino acid had mixed completely with the chemically identical compound made in the body. This assumption is certainly grossly incorrect in some cases; for example, aminolaevulic acid, if administered to an animal, behaves almost like a foreign substance; it is not used effectively for haem synthesis and is quickly excreted by the kidney. It is unlikely that amino acids arising from the hydrolysis of dietary protein behave in this extreme manner, but the assumption of a quantitative equivalence of dietary glycine or serine and the same amino acids formed in the body is not proven. This fundamental difficulty arises from the fact that the body or even the cell is not homogeneous, but consists of compartments which are semi-isolated. Thus the concentrations of different enzymes differ not only in different organs, but even in different parts of the same cell, and the rates of transfer of amino acids and other compounds across cell membranes or intracellular barriers cannot be neglected in comparison with the rates of the chemical changes under consideration (Henriques, Henriques & Neuberger, 1955). Thus a substance B, which results from the action of enzyme 1 on substance A, may be further changed into substance C by an enzyme 2 present within the same mitochondrial or microsomal structure. Substance B may normally never diffuse into the cytoplasm or be transferred into the extracellular fluid or blood plasma. If the substance B is fed to an animal or injected into an animal, its fate is likely to be completely different from that of the same substance produced and further metabolized within a particular cell structure. This difficulty is likely to be met in all experiments on whole animals, and is particularly marked if an attempt is made to interpret labelling patterns in terms of known enzyme reactions. In the present case conditions are likely to be somewhat more favourable. α-Amino acids, unlike aminolaevulic acid, are present in cells in the extracellular fluid and in the plasma in significant amounts and they appear to enter the cells of the liver, kidney and intestinal tract relatively easily. This assumption that the rate of mixing of dietary and endogenous amino acids is fast compared with the rates of metabolic reactions is likely to be incorrect, but it is probably a reasonable, if somewhat crude, approximation. The calculations made from the experimental findings indicate that the rat synthesizes about 3·5 m-moles of serine/100 g. of rat/day, whilst the corresponding value for glycine synthesis is about 2·5 m-moles. These results were critically considered by White (1958), who, using the mathematical method of Reiner (1953), obtained some-

what lower values than those given above. But the values quoted are likely to be of the correct order of magnitude. The combined daily requirements for these amino acids under conditions of maximum growth may be about 2·0–2·5 m-moles, and results obtained on effects of different doses of benzoate on growth indicate that the animal does not produce amounts of glycine greatly in excess of these requirements. In some of the experiments just discussed, labelled glycine or serine was fed as a component of diets containing homocystine, but no methionine and no choline. Growth was slow, but a comparison of the specific radioactivity of the methyl carbon atom of methionine with that of the β-carbon atom of serine allowed the conclusion to be drawn that the hydroxymethyl group of serine provides between 60 and 70 % of the methyl groups other than those already preformed in the diet. The hydroxymethyl group of serine is also likely to be the most important precursor of formate for purine synthesis.

There has been a considerable amount of discussion on the source of serine in the body, but it now seems clear that this amino acid is synthesized, not from pyruvic acid by a reversal of the serine-dehydrase reaction, but from 3-phospho-D-glycerate or glycerate through hydroxypyruvate or phosphohydroxypyruvate (Ichihara & Greenberg, 1955, 1957; Dickens & Williamson, 1959). The likely or possible reactions are shown in Fig. 5; not all enzymic steps postulated have been fully established.

Glycine is, as already discussed, formed from serine; it can be obtained from threonine by the specific aldolase enzyme and it may also arise from choline. Neither of these two last pathways is likely to be important quantitatively. The possibility has been discussed that glycine is formed from a C_2 precursor such as glyoxylate or glycollic aldehyde. The latter is transferred as a unit in the transketolase reaction, but there is no evidence that it ever appears as a free molecule in this reaction. Glyoxylic acid is formed from glycine, but its main origin in plants and micro-organisms is from D-*iso*citrate, where it functions in the glyoxylic acid cycle. The enzyme *iso*citratase, which cleaves *iso*citrate into succinate and glyoxylic acid, has never been found in animal tissues, and there is at present no suitable C_2 compound known which could form glycine by known pathways and which is made by animal cells. We must therefore assume for the present that almost all glycine synthesized is made from serine. There are thus two main groups of non-essential amino acids: the first group consists of aspartic acid, glutamic acid, asparagine, glutamine, proline, hydroxyproline and arginine, which are formed directly or indirectly from non-nitrogenous substances participating in the tricarboxylic acid cycle, namely oxaloacetate and oxoglutarate. The second group, which comprises serine, glycine and cystine, arises from 3-phosphoglycerate or D-glycerate, substances formed during glycolysis. Alanine may be considered to be a member of both groups, since pyruvic acid, its precursor, is formed in glycolysis and is also closely related to acetyl-CoA and oxaloacetate, substances which participate in the tricarboxylic acid cycle. It is of interest to note that the daily rate of alanine or pyruvate formation in the rat amounts to about 38 m-moles/100 g. (Arnstein & Grant, 1957), compared with 3·5 m-moles/100 g./day for serine, and, whilst the absolute figures are not very reliable, it is clear that the amounts of alanine, and probably those of the carbon skeletons of glutamic acid and aspartic acid, synthesized daily are about ten times those of serine and glycine.

Importance of glycine and serine in uric acid synthesis

The last problem I want to discuss is the importance of amino acids in the synthesis of uric acid as compared with that of urea. In urea synthesis carbon dioxide combines with ammonia, which is mainly derived from glutamic acid, to give carbamoyl phosphate; this combines with ornithine to give citrulline, which reacts with aspartic acid; the resulting compound is split into arginine and fumaric acid and the arginine finally yields urea and ornithine. The only two amino acids used up are aspartic acid and glutamic acid, but these can be readily regenerated by reactions which we can assume to occur at high rates in the body. Uric acid formation is likely to occur by the same mechanism as that established for inosinic acid, which has so brilliantly been elucidated in the Laboratories of J. M. Buchanan and G. R. Greenberg. In this series of reactions, two carbon atoms (C-2 and C-8) come from formate through a tetrahydrofolic acid derivative and two carbon atoms (C-4 and C-5) originate from glycine, whilst C-6 is derived from carbon dioxide. Of the four nitrogen atoms, one (N-7) is specifically derived from glycine, whilst the other three originate from aspartic acid and glutamine. The semi-essential character of glycine for the young chick suggests that the capacity of the bird to synthesize glycine is, like

L-Serine ⟷ hydroxypyruvate ← D-glyceric acid
⇅ ⇅ ⇅
Phospho-L-serine ⇋ 3-phosphohydroxypyruvate ← 3-phosphoglycerate

Fig. 5. Possible pathways of the biosynthesis of serine (following Greenberg).

that of the rat, not very great, at least not as great as the ability to form glutamic acid or aspartic acid. Moreover, it is likely that in the chicken, as in the rat, C_1 units are mainly derived from the β-carbon atom of serine, and it appears therefore that in the bird one or two molecules of serine or glycine have to be supplied in the diet or synthesized in the body in order to excrete three nitrogen atoms in the form of uric acid. It follows therefore that nitrogen excretion in uricotelic animals is dependent on the rate of synthesis of glycine and serine, substances formed from intermediates in glycolysis and probably only available in limited amounts. Urea synthesis, on the other hand, depends on the availability of aspartic acid and glutamic acid, substances which can be made by the organism in large amounts, and which are derived from tricarboxylic acid-cycle intermediates. Uric acid synthesis also differs from urea synthesis in the demands made by the former on an adequate supply of folic acid. These considerations may help to explain some of the specific nutritional requirement of uricotelic animals.

An attempt has been made in this lecture to weave together results obtained by methods of organic chemistry and observations made with isolated enzymes, and I have tried to use such results to interpret experiments done with the whole animal. I am conscious that I have not fully achieved my aim, but I hope to have given at least some indication as to how further progress can be made.

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An Investigation of the Egg-White Mucoproteins, Ovomucoid and Ovalbumin

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Very little information is available about the structure of the mucoproteins because of the difficulties that arise when classical methods are applied. The severe difficulties in the preparation of workable quantities of pure mucoproteins has also retarded structural studies. Only the carbohydrate of ovomucoid has received a detailed structural investigation (Stacey & Woolley, 1940, 1942). This substance contains about 25% of carbohydrate, made up of D-mannose, D-galactose and 2-acetamido-2-deoxy-D-glucose units, and is readily prepared, and so forms an excellent model substance for investigation of the mucoproteins in general. Ovalbumin, although containing less than 5% of carbohydrate, composed of D-mannose and 2-acetamido-2-deoxy-D-glucose (Neuberger, 1938), may be obtained in a greater state of purity than ovomucoid. The nature of the carbohydrate peptide bond remained obscure until recent examination of the glycopeptides produced on enzymic hydrolysis of ovalbumin suggested that aspartic acid is directly linked to the carbohydrate through one of its carboxyl groups (Cunningham, Neunke & Neunke, 1957; Jevons, 1958; Johanson, Marshall & Neuberger, 1958). This paper describes some attempts to enrich the carbohydrate component of these mucoproteins by treatment with alkali and by other methods, but in no case was the pure carbohydrate isolated. Application of methods commonly employed in polysaccharide chemistry to the structural studies of mucoproteins has been studied with reference to ovomucoid and ovalbumin. As a result some new structural features of these mucoproteins have been revealed.

EXPERIMENTAL

Preparation of the mucoproteins

Ovomucoid. Powdered egg albumin (British Drug Houses Ltd.; 750 g.) was mechanically stirred for 6 hr. with water (25 l.). The addition of 10% (w/v) trichloroacetic acid (3 l.) produced a copious white precipitate which was removed on the centrifuge (approx. 600 *g*/30 min.) and the supernatant liquid was dialysed for several days. The clear-yellow liquid, obtained after filtration, was concentrated at a temperature below 35° to a smaller volume (3·5 l.). A precipitate of crude ovomucoid was obtained by the addition of methylated spirits (6·6 l.) at −10°. Several reprecipitations from aqueous solution with 2 vol. of ethanol at −10° afforded, on drying under reduced pressure, a white powder (yield, 20 g.); $[\alpha]_D^{25}$ −62±3° in water (*c*, 1·02) [Found, sulphated ash, 2·8; N (Dumas), 13·4; P, nil; acetyl, 3·8; OMe, 0·2%].

Ovalbumin. Crystalline ovalbumin was prepared from egg albumin (British Drug Houses Ltd.) by Kekwick & Cannan's (1936) procedure and recrystallized twice. Dialysis of an aqueous solution of the crystals to remove sodium sulphate, followed by freeze-drying, yielded a white, easily water-soluble solid [Found: sulphated ash, 3·7; N (Dumas), 14·9%].

Analysis of the mucoproteins

Electrophoresis. The mucoproteins were examined in the Tiselius apparatus (Hilger and Watts Ltd.). Solutions (1%, w/v) in the appropriate buffer solution were equilibrated by dialysis against a large volume (> 2 l.) of the same buffer at 4° for 24 hr. Electrophoresis was then done at 4° with a current of 10–15 mA in one of the following buffers: phosphate, pH 6·9, *I* 0·13; acetate, pH 4·4, *I* 0·01. Electrophoresis of ovomucoid was also carried out in 0·2 M-KH_2PO_4–0·1 M-NaOH buffer, pH 6·8, in the presence of guanidine hydrochloride (50 g./l.) and sodium chloride (15 g./l.).

Paper electrophoresis was performed on Whatman no. 1 paper soaked in 0·025 M-sodium tetraborate, pH 9, for 3 hr. at 5 mA. The mucoproteins were detected by the bromophenol blue reagent (Durrum, 1950).

Absorption spectra. The ultraviolet absorption of ovomucoid was measured at 0·1% concentration in water, with a Unicam SP. 500 spectrophotometer and a 1 cm. cell.

Paper chromatography. Paper chromatography was carried out by the descending method on Whatman no. 1 filter paper with the following solvent systems: (1) butan-1-ol–ethanol–water (40:11:19, by vol.); (2) butanol–pyridine–water (10:3:3, by vol.); (3) ethyl acetate–acetic acid–water (9:2:2, by vol.); (4) benzene–ethanol–water (169:47:15, by vol; top layer); (5) ethyl acetate–acetic acid–formic acid–water (18:3:1:4, by vol.). Aldoses and methylated aldoses were detected by *p*-anisidine hydrochloride (Hough, Jones & Wadman, 1950), lactones by the method of Abdel-Akher & Smith (1951), 2-amino-2-deoxyhexoses by spray reagents based on the Elson–Morgan test (Partridge, 1948) and polyols with the periodate–*p*-anisidine spray (Bragg & Hough, 1958).

Characterization of the monosaccharide components

Ovomucoid. Acid hydrolysis with 2 N-H_2SO_4 for 48 hr. at 100° and subsequent paper-chromatographic examination indicated the presence, in unequal amounts, of galactose,

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mannose and 2-amino-2-deoxyglucose, together with amino acids. The neutralized hydrolysate ($BaCO_3$) was freed of amino acids and the amino sugar by passage down a cation-exchange column [Amberlite IR-120 (H)]. After concentration of the eluate D-mannose and D-galactose were separated by paper chromatography (solvent 3) and then characterized as the phenylhydrazone (m.p. and mixed m.p. 191–193°) and the 1-methyl-1-phenylhydrazone (m.p. and mixed m.p. 181°) derivatives respectively (Hirst, Jones & Woods, 1947).

A similar hydrolysate of ovomucoid (1 g.) was passed through a squat charcoal column (Andrews, Hough & Powell, 1956) and the column was thoroughly washed with water. The effluent and combined washings were acidified with concentrated HCl (1 ml.) and concentrated to yield 2-amino-2-deoxy-D-glucose hydrochloride, which after recrystallization from aq. ethanol had $[\alpha]_D + 70°$ in water (12 hr.; *c*, 0·43). The absence of other amino sugars in ovomucoid was shown by ninhydrin oxidation (Ruelius & Girard, 1954) of the hydrolysate, when only arabinose was found.

Ovalbumin. The mucoprotein (100 g.) was hydrolysed in 2N-H_2SO_4 (500 ml.) at 95–100° for 48 hr. After neutralization ($BaCO_3$), the solution was adjusted to pH 4 and passed through a cation-exchange column [Amberlite IR-120 (H)]. Concentration of the effluent gave a syrup which on heating with an ethanolic solution of phenylhydrazine gave D-mannose phenylhydrazone (0·5 g.) with m.p. and mixed m.p. 190–193° (Hirst *et al.* 1947). The phenylhydrazone (0·3 g.) was heated under reflux with an excess of benzaldehyde in ethanol–water (3:1, v/v) until all the crystals had dissolved. The reaction mixture was exhaustively extracted with ether to remove excess of benzaldehyde and its phenylhydrazone derivative. The aqueous solution was concentrated to a syrup (160 mg.), which had $[\alpha]_D^{25} + 8°$ in water (*c*, 3·2) and was indistinguishable from mannose on paper chromatograms.

Ninhydrin oxidation of a small quantity of the above-mentioned ovalbumin hydrolysate, followed by paper chromatography, revealed arabinose as the only pentose component, in agreement with the occurrence of 2-amino-2-deoxy-D-glucose in this mucoprotein (Neuberger, 1938).

Quantitative analysis for neutral monosaccharides

Resin-hydrolysis method. Cation-exchange resin (Amberlite IR-120: 10 g.) was shaken for 30 min. with 10% HCl (40 ml.) and washed with water until the washings were free from Cl^- ions. The resin was dried by washing successively with ethanol and ether. The mucoprotein (approx. 50 mg.) was accurately weighed into a small test tube (9 cm. × 1 cm.) and water (2·5 ml.) and prepared ion-exchange resin (1 g.) were added. The tube was closed with a rubber cap and heated at 95–100° for 24–30 hr. The pressure that developed during the initial stages of heating was released by puncturing the cap with a hypodermic needle. After hydrolysis a known weight (5–10 mg.) of either ribose or xylose was added to the cooled hydrolysate, the resin was filtered off and the monosaccharides were separated on paper chromatograms (Flood, Hirst & Jones, 1948) for subsequent determination by Jones & Pridham's (1954) benzidine method.

Acid-hydrolysis method. The mucoprotein (approx. 50 mg., accurately weighed) was hydrolysed with 2N-H_2SO_4 (5 ml.) in a sealed tube at 95–100° for 18 hr. A weighed quantity of ribose or xylose was then added and the solution was neutralized ($BaCO_3$). After filtration, the hydrolysate was slowly passed down a cation-exchange column [Amberlite IR-120 (H); 10 cm. × 1 cm.], which was then washed with water until the effluent gave a negative Molisch test. The total effluent was concentrated under reduced pressure, the monosaccharide components were separated by paper chromatography and then determined as described above. Quantitative estimates of the monosaccharide components of ovalbumin and ovomucoid are shown in Table 1.

Quantitative estimation for 2-amino-2-deoxyglucose. The mucoprotein (approx. 50 mg., accurately weighed) was hydrolysed with 2N-H_2SO_4 (2·5 ml.) at 95–100° in a sealed glass tube. Samples were removed at various times up to 48 hr. and, when cool, were neutralized with 2N-NaOH, made up to 100 ml. and amino sugar was determined by the method of either Belcher, Nutten & Sambrook (1954) or Tracey (1952). Results are shown in Table 1.

Table 1. *Carbohydrate composition of ovomucoid and of ovalbumin*

Mannose and galactose were determined by the methods described in the text, after hydrolysis of the mucoproteins with cation-exchange resin, followed by separation of the sugars by paper chromatography. The hydrolysis of each sugar was followed until a constant value was obtained.

Mucoprotein	Amount found (% of hexose or amino hexose)		Molecular ratio
Ovomucoid	D-Mannose	6·3 ± 0·3	3·7
	D-Galactose	1·7 ± 0·3	1
	2-Amino-2-deoxy-D-glucose (method of Belcher, Nutten & Sambrook, 1954)	12·3 ± 0·5	7·2
	2-Amino-2-deoxy-D-glucose (method of Tracey, 1952)	12·8 ± 1·7	7·4
Ovalbumin	Mannose	2·75 ± 0·15	2
	2-Amino-2-deoxy-D-glucose (method of Belcher *et al.* 1954)	1·34 ± 0·04	1

Structural investigations of the mucoproteins

Acid-hydrolysis studies on ovomucoid. By following the rate at which the components of a polysaccharide appear during acidic hydrolysis useful structural data can often be obtained about the monosaccharides which occupy outer and inner positions in the macromolecule, as, for example, with the blood-group polysaccharides (Kabat & Leskowitz, 1954).

A solution of ovomucoid (57 mg.) in water (5 ml.) was heated (95–100°) with ion-exchange resin [Amberlite IR-120 (H); 1 g.] in a sealed, all-glass polarimeter tube (0·535 dm. in length) and the change in optical rotation was followed to a constant value (Table 2). In a similar experiment the liberated D-mannose and D-galactose were determined quantitatively, as described above, by separation on paper chromatograms followed by estimation of the monosaccharides by the benzidine method (Jones & Pridham, 1954) (see Table 2).

In another experiment ovomucoid (60 mg.) was heated at 100° with 2N-H_2SO_4 (2·5 ml.) and at various intervals of time the appearance of reducing sugar in the hydrolysate was measured (Somogyi, 1945) and the corresponding liberation of 2-amino-2-deoxy-D-glucose determined by the method of Belcher *et al.* (1954) (Table 3).

Table 2. *Rate of appearance of* D-*mannose and* D-*galactose, and the change in optical rotation, when ovomucoid was heated in aqueous solution with cation-exchange resin*

The sugars were determined colorimetrically (Jones & Pridham, 1954) after quantitative separation on paper chromatograms.

Duration of heating (hr.)	Mannose (%)	Galactose (%)	Optical rotation ($\alpha°$)
0	—	—	−0·29
½	2·1	1·6	−0·29
3	2·6	1·6	—
6	2·3	1·6	—
10·5	—	—	−0·04
12	5·0	1·6	—
24	5·2	1·6	+0·02
30	—	—	+0·03
48	6·0	1·6	+0·03
70	—	—	+0·04

Table 3. *Change in reducing power and liberation of 2-amino-2-deoxy-*D*-glucose during acid hydrolysis of ovomucoid*

Ovomucoid was hydrolysed for various times at 100° with 2N-H_2SO_4 and the liberated 2-amino-2-deoxyglucose (Belcher *et al.* 1954) and increase in reducing power (Somogyi, 1945) were determined.

Duration of hydrolysis (hr.)	Reducing power (% of hexose)	2-Amino-2-deoxy-D-glucose (%)
1	17·4	8·6
3	22·2	11·4
6	21·2	12·1
12	23·2	12·8
24	21·0	12·0
48	—	10·1

Since monosaccharides which are linked in the furanose form are readily hydrolysed by 0·01 N-H_2SO_4 at 80°, whereas pyranosides are not so susceptible (Andrews *et al.* 1956), the hydrolysis products of ovomucoid at pH 2·0 and 80° were investigated on paper chromatograms, but no monosaccharides were rapidly liberated under these conditions.

The isolation and characterization of disaccharides and other oligosaccharides from partial hydrolysates of mucoprotein can afford conclusive evidence of the interglycosidic linkages. This type of linkage analysis was applied to ovomucoid. Difficulties due to the presence of large quantities of peptides and amino acids, and to low yields of oligosaccharides, were encountered. Ovomucoid (6 g.) was heated at 95–100° with N-H_2SO_4 (200 ml.) for 6 hr.: previous examination of a similar hydrolysate had suggested that this time gave the optimum yield of oligosaccharides. The neutralized [$Ba(OH_2)$] liquid was filtered, concentrated to about 100 ml. and separated by adsorption on to a squat charcoal column followed by fractional elution with ethanol–water mixtures of increasing ethanol content (Andrews *et al.* 1956).

An amorphous disaccharide (11·8 mg.; R_{Gal} 0·39, solvent 5) was obtained on elution with aq. 5·0–7·5% (v/v) ethanol. It had $[\alpha]_D$ +16° in water (*c*, 1·18), contained traces of a contaminating amino acid and gave only mannose on acid hydrolysis. Oxidation of this crude disaccharide with sodium metaperiodate in unbuffered solution and at pH 8·0 (Hough & Perry, 1956) gave 0·57 mole (8 hr.) and 0·9 mole (5 hr.) of formaldehyde respectively.

Partial hydrolysis of ovalbumin with acid. A suspension of denatured ovalbumin (80 g.) in water (2 l.) was taken to pH 1 by addition of trichloroacetic acid and heated at 80° for 12 hr. with continuous stirring; the pH had then risen to 7. After filtration, the clear solution was evaporated to a solid, which was extracted with 80% (v/v) methanol–water. The extract was examined on paper chromatograms but no free sugars were detected. The residue was soluble in water and had $[\alpha]_D$ −26° (*c*, 1·04) (Found: sulphated ash, 50·2; N, 7·1%). Paper ionophoresis in 0·05 M-sodium tetraborate for 13 hr. at 3 mA showed the presence of a major component which had moved 6·0 cm. towards the anode and gave a positive reaction with the periodate spray reagent; on paper chromatograms it remained on the starting line. Acid hydrolysis with N-H_2SO_4 followed by paper chromatography revealed the presence of mannose (4·7%), 2-amino-2-deoxy-D-glucose (5·3%) and amino acids, including glutamic acid and aspartic acid, glycine and alanine. An *N*-terminal end-group analysis (Partridge & Davis, 1955; Blackburn & Lowther, 1951) showed only *N*-dinitrophenylalanine on paper chromatograms.

Periodate oxidation of the mucoproteins

Valuable evidence for carbohydrate structures has been obtained by periodate oxidation and exhaustive methylation, and in the vast majority of cases these methods have provided mutually confirmatory results. The periodate method has the advantage that it can be applied directly and requires very mild conditions. However, the presence of protein can interfere with the determination of the formic acid produced from the carbohydrate (Anderson, Greenwood & Hirst, 1955) and the uptake of periodate (Bragg & Hough, 1958). Consequently the main use of this method when applied to mucoproteins is to determine quantitatively which monosaccharide units are attacked by periodate.

Table 4. *Periodate oxidation of ovomucoid at different* pH *values*

Ovomucoid (100 mg.) was dissolved in water or buffer solution (100 ml.). Portions (5 ml.) were removed at various times and the consumption of periodate (Neumüller & Vasseur, 1953) and production of acid (Anderson, Greenwood & Hirst, 1955) were determined.

Time of reaction (hr.)	Consumption of periodate (g. of ovomucoid/mole of $NaIO_4$)			Acid production (g. of ovomucoid/equiv. of acid)	
	Unbuffered	pH 6·96	pH 3·5	Indicator	Potentiometer
0·5	—	1580	1359	—	—
1	1033	1295	1045	40 300	41 700
1·5	—	1154	—	—	—
2	1009	1095	847	20 750	41 700
3	916	1067	847	—	—
4	—	—	788	10 075	—
5	790	—	—	—	—
6	—	1040	—	—	—
7	720	—	663	8 060	41 700
9	630	710	—	—	—
24	438	418	494	4 030	10 410
48	353	—	400	—	—

Table 5. *Composition of the products after periodate oxidation of ovomucoid and ovalbumin*

Ovomucoid (or ovalbumin) was oxidized by periodate for 2 hr. in the dark. Residual periodate was then destroyed with excess of ethylene glycol. Ions were removed by dialysis (2 days) and the product was hydrolysed with 2 N-H_2SO_4. The liberated monosaccharides were determined by the methods described in the text.

Aldose	Found (%)
Ovomucoid	
D-Mannose	3·2
D-Galactose	0
2-Amino-2-deoxy-D-glucose	5·9
Ovalbumin	
D-Mannose	0·85
2-Amino-2-deoxy-D-glucose	0·92

Table 6. *Consumption of periodate by ovalbumin at* pH 3·6

Ovalbumin (430 mg.) was dissolved in acetate buffer (pH 3·6; 50 ml.), 0·3 M-sodium metaperiodate (10 ml.) was added and the solution made up to 100 ml. with water. Portions (5 ml.) were removed for the determination (Neumüller & Vasseur, 1953) of periodate consumption.

Time of reaction (hr.)	Consumption of periodate (g. of ovalbumin/mole of $NaIO_4$)
0·5	1270
1	1162
2	1195
3	1180
6	985
17	686
42	489

The oxidations were investigated at both pH 3·6 and pH 6·96 to diagnose any over-oxidation of the carbohydrate, since this can have structural significance. Intermediary formyl esters formed by oxidative cleavage of the $C_{(1)}$–$C_{(2)}$ bond of the reducing end group are relatively stable at pH 3·6 but labile at pH 6·96, and consequently lead to further oxidation at the latter pH through the oxidation of malondialdehyde and other derivatives (Hough & Perry, 1956; Hough, Perry & Woods, 1957; Hough, Taylor, Thomas & Woods, 1958; Cantley, Hough & Pittet, 1959). The course of the periodate reaction was also followed in unbuffered solution to determine the acid liberated in relation to the amount of periodate that had reacted.

Ovomucoid. Ovomucoid (100·9 mg.) was dissolved in water (90 ml.) and 0·3 M-sodium metaperiodate solution (5 ml.) was added. The solution was made up to 100 ml. and transferred to a brown bottle (Potter & Hassid, 1948). A blank was prepared by omission of the ovomucoid. At various times, portions (10 ml.) were transferred into a mixture of 0·2 M-Na_2HPO_4–0·13 M-KH_2PO_4 buffer (pH 7·0; 20 ml.) and 20% KI (3 ml.) (Neumüller & Vasseur, 1953). The liberated iodine was titrated with 0·01 N-$Na_2S_2O_3$ with 1% starch as indicator and from this figure the periodate uptake was determined.

Similar determinations at different pH values were carried out by dissolving the mucoprotein in sodium phosphate buffer (pH 6·96; 25 ml.) and acetate buffer (pH 3·5; 25 ml.) respectively and then proceeding as described above by dilution to 100 ml. after addition of 0·3 M-sodium metaperiodate solution (5 ml.). The results are shown in Table 4. The release of formic acid and formaldehyde was then investigated; ovomucoid (0·201 g.) was dissolved in water (100 ml.) containing 0·3 M-sodium metaperiodate (5 ml.) and transferred to a brown bottle. A blank was prepared by omission of the mucoprotein. Samples (10 ml.) were mixed with ethylene glycol (5 ml.) and, after keeping for 10 min., the acid was titrated with 0·01 N-NaOH, either potentiometrically to pH 6·25 (Anderson *et al.* 1955) or with methyl red–methylene blue indicator (0·1%; 4:1, v/v) (Table 4). Tests for formaldehyde, by using Schryver's (1910) reagents on the final reaction solutions, gave negative results.

To determine which monosaccharides had been attacked by periodate, ovomucoid was allowed to react for 2 hr. with periodate, under the unbuffered conditions described above, and then the excess of periodate was destroyed by the addition of an excess of ethylene glycol. After dialysis for 2 days to remove sodium iodate, formaldehyde, formic acid and excess of glycol, the oxidized mucoprotein was isolated by evaporation under reduced pressure and hydrolysed with acid, and the liberated monosaccharides were determined as

described above for the mucoprotein. The results are in Table 5.

Ovalbumin. The methods employed were similar to those used for ovomucoid. No ammonia and a negligible quantity of acid was produced during the oxidation under unbuffered conditions. For quantitative results see Tables 5 and 6.

Exhaustive methylation of the mucoproteins and examination of the components of the methylated products

Whilst the methylation technique has been widely applied in the structural determination of polysaccharides and the mucopolysaccharides, the only mucoproteins so investigated have been ovomucoid (Stacey & Woolley, 1940, 1942) and frog-spawn mucin (Folkes, Grant & Jones, 1950). In both cases extensive degradation was encountered during the reaction with dimethyl sulphate and strong aqueous alkali (as a consequence of the presence of protein moiety and the amino sugar). As a preliminary to this investigation alternative techniques were examined. However, sodium in liquid ammonia, followed by treatment with methyl iodide (Freudenberg, Plankenhorn & Boppel, 1938; Hodge, Karjala & Hilbert, 1951), and another method involving treatment with thallous hydroxide followed by methyl iodide (Campbell, Hirst & Jones, 1948), were both unsuccessful. Consequently recourse was had to a modification of the dimethyl sulphate method described by Stacey & Woolley (1940, 1942).

Methylation of ovomucoid. A mixture of ovomucoid (25 g.), water (100 ml.), carbon tetrachloride (300 ml.) and dimethyl sulphate (100 ml.) was stirred whilst 35% (w/v) sodium hydroxide solution (200 ml.) was added dropwise over 2 hr. After another hour, dimethyl sulphate (100 ml.) and 35% (w/v) sodium hydroxide solution (200 ml.) were added simultaneously and dropwise over a period of 2 hr. The reaction mixture was stirred for a further 24 hr., when the insoluble material (A) was filtered off, the filtrate was neutralized with acetic acid, concentrated to dryness and the residue was exhaustively extracted with methanol. After evaporation of the methanol, the residue was dissolved in aq. 50% (v/v) acetone (400 ml.), mixed with material A and methylated twice as before. The reaction mixture was neutralized with acetic acid, evaporated to dryness and continuously extracted with acetone for 24 hr. The acetone extract was concentrated, the residue was extracted with chloroform and the soluble material isolated as a thick syrup by evaporation of the solvent. The product (4·6 g.) had $[\alpha]_D^{25} - 8{\cdot}3°$ in water (*c*, 1·21) (Found: sulphated ash, 8·4; N, 6·0; OMe, 19·3%).

This methylated product was treated with acetic anhydride for 15 hr. to acetylate any free amino groups, and then, after removal of acetic anhydride by distillation, was heated under reflux with methyl iodide in the presence of silver oxide (Bray, Henry & Stacey, 1946). Finally, the reaction mixture was filtered, H_2S was passed through the solution to remove Ag^+ ions, the mixture was filtered again and the filtrate evaporated to dryness. The resultant syrup (2·9 g.) had $[\alpha]_D^{25}$ 0° in acetone (*c*, 0·78) (Found: sulphated ash, 10·8; N, 5·2; OMe, 7·9%).

Methylation of ovalbumin. Denatured ovalbumin (300 g.) was methylated by repeated treatments with dimethyl sulphate and 35% (w/v) NaOH solution, in the same manner as described for ovomucoid, to give an acetone-soluble product (30 g.) (Found: sulphated ash, 38·2; N, 3·7; OMe, 11%). After *N*-acetylation with acetic anhydride and subsequent treatment with silver oxide and methyl iodide, an aqueous solution of the product (21 g.) was deionized (Bio-Deminrolit) and evaporated to give a thick syrup (6·5 g.) which had $[\alpha]_D^{25} - 14°$ in water (*c*, 0·98) (Found: sulphated ash, 3·8; N, 10·3; OMe, 12·5%).

Examination of the methylated sugars produced on hydrolysis of the methylated ovomucoid fraction. A solution of the methylated ovomucoid fraction (2·8 g.) in methanolic 4% (w/v) HCl was heated under reflux for 5 days. The solution was neutralized (Ag_2CO_3) and Ag^+ ions were precipitated

Table 7. *Fractionation of hydrolysate of methylated ovomucoid*

The hydrolysate of methylated ovomucoid (2·8 g.) was adsorbed on a charcoal column. The methyl sugars were eluted by fractional elution with ethanol–water mixtures.

Concn. of ethanol (%)*	Wt. (mg.)	$[\alpha]_D$ (water)	Solvent I R_F	Solvent I R_G	Comments	Methylated sugars present
Water	360	—	—	—	Amino acids present	—
2·5	248	—	—	—	Amino acids present	—
5	170	—	0·54 0·36	0·64 0·54	Contained amino acids	Di-*O*-methylmannose; mono-*O*-methylmannose
10	176	—	0·54 0·36	0·64 0·54	Contained amino acids	Di-*O*-methylmannose; mono-*O*-methylmannose
15	109	+32°	0·63 0·57	0·76 0·69	Faster sugar predominates	Tri-*O*-methylhexoses
20	76	+43°	0·70 0·61	0·84 0·74	80% of faster sugar —	2:3:4:6-Tetra-*O*-methyl-D-galactose Tri-*O*-methylhexose
25	98	+36°	0·81 0·74	0·97 0·84	Slower sugar predominated (3:1) —	2:3:4:6-Tetra-*O*-methyl-D-mannose 2:3:4:6-Tetra-*O*-methyl-D-galactose
30	30	—	0·81	0·97	—	2:3:4:6-Tetra-*O*-methyl-D-mannose
40	31	+48°	0·75	0·92	—	—

* Each fraction was eluted with 1 l. of solvent.

with H_2S. After filtration the solution was concentrated to a syrup containing the methyl glycosides, which were hydrolysed for 3 hr. with N-H_2SO_4 at 95–100°. The hydrolysate was neutralized ($BaCO_3$) and the mixture of methylated sugars fractionated by adsorption on to a squat column of acid-washed charcoal (4 cm. × 10 cm.; British Drug Houses Ltd.) followed by fractional elution with increasing concentrations of ethanol in water (Andrews *et al.* 1956). The results are summarized in Table 7. This technique was favoured to partition chromatography on a cellulose column since experience had shown that the charcoal column gave fractions which were less contaminated by amino acids. Each fraction (1 l.) from the column was deionized (Bio-Deminrolit) before evaporation, since they were slightly acidic, and then was examined on paper chromatograms. The water washings from the column were carefully examined for mannose but none was found.

Paper chromatography of the fractions eluted with aq. 5 and 10% (w/v) ethanol suggested that they contained the same two methylated sugars. The fractions were combined and the two components separated on paper chromatograms (solvent 3). Both methylated sugars yielded mannose on de-*O*-methylation with hydrobromic acid (46%, w/v) at 100° for 6 min. (Hough *et al.* 1950). Their rates of movement on paper chromatograms were typical of a mono-*O*-methylmannose and a di-*O*-methylmannose respectively. An attempt to isolate a phenylosazone derivative of the mono-*O*-methylmannose (19 mg.) was unsuccessful. The faster-moving di-*O*-methyl component (21 mg.) had $[\alpha]_D^{25} + 13°$ in water (*c*, 0·35). This reducing sugar was oxidized with sodium metaperiodate for 5 hr., as described by Hough, Powell & Woods (1956), and the reaction mixture was examined for formaldehyde but none was found. Similarly, no formaldehyde was detected after reduction of the di-*O*-methylmannose with sodium borohydride to a di-*O*-methylmannitol derivative followed by periodate oxidation (Hough *et al.* 1957). This evidence suggests that one of the components from hydrolysed methylated ovomucoid is 2:6-di-*O*-methyl-D-mannose.

The fraction eluted with aq. 15% (v/v) ethanol was obtained as a syrup (109 mg.) (Found: OMe, 40·6; N, 0·5. Calc. for tri-*O*-methylhexose: OMe, 41·8%). Oxidation of this syrup with bromine–water gave a mixture of lactones, as revealed by paper chromatography (solvents 1 and 4), which we failed to characterize.

The fraction eluted with aq. 20% (v/v) ethanol was obtained as a syrup (76 mg.) (Found: OMe, 45·1; N, 0·7. Calc. for tetra-*O*-methylhexose: OMe, 52·5%), which was heated under reflux for 3½ hr. with redistilled aniline (34 mg.) in ethanol (5 ml.). Evaporation of the reaction mixture yielded needle-shaped crystals with $[\alpha]_D - 78° \rightarrow +35°$ (equil.) in acetone (*c*, 0·4) and m.p. and mixed m.p. with authentic 2:3:4:6-tetra-*O*-methyl-D-galactose anilide, 188° [Found: OMe, 35·9. Calc. for $C_{12}H_{12}ON(OMe)_4$: OMe, 39·9%].

The 25% (v/v) ethanol fraction contained a mixture of tetramethyl sugars (Found: OMe, 48·9; N, 0·8. Calc. for tetramethylhexose: OMe, 52·5%), which were separated by paper chromatography (solvent 1) and converted into 2:3:4:6-tetra-*O*-methyl-D-mannose anilide, m.p. and mixed m.p. 147°, and 2:3:4:6-tetra-*O*-methyl-D-galactose anilide, m.p. and mixed m.p. 188° respectively. Present in the 30% (v/v) ethanol fraction was a tetra-*O*-methyl sugar, which was converted, as described above, into the anilide, which had $[\alpha]_D - 10°$ (equil.) in methanol (*c*, 0·1) and m.p. and mixed m.p. with authentic 2:3:4:6-tetra-*O*-methyl-D-mannose anilide, 147–148° [Found: OMe, 38·3. Calc. for $C_{12}H_{12}ON(OMe)_4$: OMe, 39·9%].

The fraction eluted by aq. 40% (v/v) ethanol consisted largely of methyl glycosides, since hydrolysis with 2N-HCl for 10 hr. at 100° produced a mixture of methyl sugars consisting largely of tetra-*O*-methylmannose, with tetra-*O*-methylgalactose and an unidentified tri-*O*-methylhexose as minor components.

Examination of the methylated sugars produced on hydrolysis of the methylated ovalbumin fraction. The methods employed are the same as those used above for methylated ovomucoid. The methylated ovalbumin (6·5 g.) was heated under reflux in methanolic 4% (w/v) HCl (170 ml.) for 3 days and the liberated methyl glycosides were then hydrolysed for 3 hr. at 95–100° with N-H_2SO_4 (25 ml.). After neutralization ($BaCO_3$), the neutral solution was evaporated to a syrup (4·8 g.), which was extracted by heating under reflux with chloroform and with methanol to enrich the methylated sugars. The combined extracts were evaporated to a syrup (1·43 g.), which was fractionated on a column of acid-washed charcoal (10 cm. × 5 cm.; British Drug Houses Ltd.) by fractional elution with water–ethanol mixtures (Table 8).

All fractions were observed to contain amino acids. Evaporation of the aq. 10% (v/v) ethanol fraction gave a syrup (40 mg.) which had the mobility of a di-*O*-methylmannose on paper chromatograms. No formaldehyde was produced on periodate oxidation of the sugar either before

Table 8. *Fractionation of hydrolysate of methylated ovalbumin*

The hydrolysate was adsorbed on a charcoal column followed by fractional elution with ethanol–water mixtures.

Concn. of ethanol (%)*	Wt. of fraction (mg.)	Solvent I R_F	Solvent I R_G	Methylated sugar present
Water	14	—	—	—
2·5	17	—	—	—
5	50	0·29	—	—
—	—	0·33	—	—
10	40	0·57	0·68	Di-*O*-methylmannose
20	51	0·71	0·81	Tri-*O*-methylmannose
30	72	0·71	0·81	Tri-*O*-methylmannose
40	83	0·82	0·98	2:3:4:6-Tetra-*O*-methyl-D-mannose
50	66	0·82	0·98	2:3:4:6-Tetra-*O*-methyl-D-mannose

* Each fraction was eluted with 1 l. of solvent.

Table 9. *Composition of the fractions formed by the action of barium hydroxide on ovomucoid*

See text for details of preparation of the fractions and method of analysis.

Fraction	$[\alpha]_D$ (water)	N (%)	Sulphated ash (%)	2-Amino-2-deoxy-D-glucose (%)	Mannose (%)	Galactose (%)	Ratio of mannose:amino sugar
I*	13±2°	4·2	0·2	21·2	+	+	—
I†	—	—	—	9·2	17	0	—
IA	—	—	1·9	17	19	10	1·1
IB	—	—	0·8	22·5	28	38	1·25
II	16±4°	—	8·8	12	12	5	1·0
III	10°	—	0·7	0·9	1·2	1·8	1·3
IV	—	—	3·1	6·0	7·0	5·0	1·2

* Acetyl absent. † After oxidation with periodate.

or after reduction with sodium borohydride (Hough *et al.* 1957). This evidence suggested that the sugar was 2:6-di-*O*-methyl-D-mannose.

The syrup (79 mg.) obtained by evaporation of the aq. 40 and 50% (v/v) ethanol fractions was indistinguishable from 2:3:4:6-tetra-*O*-methylmannose on paper chromatograms. It had $[\alpha]_D^{25}$ +25·5° in methanol (*c*, 0·47) and was converted into the crystalline anilide, which had m.p. and mixed m.p. 145–147° with authentic 2:3:4:6-tetra-*O*-methyl-D-mannose anilide.

Attempts to enrich the carbohydrate moiety of the mucoproteins

Several authors have claimed to have isolated carbohydrate fractions from mucoproteins by the action of aq. $Ba(OH)_2$ (Levene & Mori, 1929; Fränkel & Jellinek, 1927; Neuberger, 1938; Stacey & Woolley, 1940). It was claimed that the protein was degraded by alkaline hydrolysis, but that the carbohydrate was, in the main, stable to alkali. However, useful structural information has been obtained by studying the action of aqueous alkali on polysaccharides (Kenner, 1955), since 1 → 3-linked and 1 → 4-linked hexose polymers undergo stepwise degradation from the reducing end group along the polysaccharide chain, with the formation, and peeling off, of a saccharinic acid unit from each hexose unit, whereas 1 → 2 or 1 → 6 linkages impede this degradation process. The action of aq. $Ba(OH)_2$ on ovomucoid and ovalbumin was studied further to determine if there had been any loss of carbohydrate by this chain-degrading process.

Ovomucoid. A mixture of ovomucoid (10 g.), 10% (w/v) $Ba(OH)_2$ soln. (150 ml.), ethanol (15 ml.) and octan-2-ol (10 ml.) was heated under reflux for 17 hr. in a stream of nitrogen. After neutralization with H_2SO_4, the filtered solution was evaporated to approx. 40 ml. and poured into an excess of methylated spirits. The light-brown precipitate was centrifuged off, dissolved in water, reprecipitated with ethanol and dried. The supernatant ethanol was found to contain only amino acids, and no carbohydrates could be detected even after hydrolysis (2N-H_2SO_4). The precipitated material was non-reducing to Fehling solution and, on hydrolysis with N-H_2SO_4, gave amino acids, mannose, galactose and 2-amino-2-deoxyglucose. In a quantitative experiment there were found 9·5% of mannose, 7·6% of galactose and 7% of 2-amino-2-deoxyglucose.

In a large-scale experiment ovomucoid (70 g.) was degraded as described above and the cooled solution acidified with H_2SO_4 and then neutralized ($BaCO_3$). After filtration, the solution was concentrated almost to dryness and the thick syrup poured into ethanol (1 l.). The precipitated solid was isolated by centrifuging, dissolved in water (200 ml.) and ethanol (200 ml.) was added. The precipitate which formed was removed by centrifuging. The supernatant liquid was poured into ethanol (1·6 l.) to yield a light-brown solid (9 g.) (Found: sulphated ash, 32·1; N, 7·0%). Hydrolysis of a small portion revealed galactose, mannose, 2-amino-2-deoxyglucose and small amounts of amino acids. A solution of the solid was deionized by passage through a column of ion-exchange resin [Amberlite IR-120 (H)] to give an acidic solution, which was poured into ethanol (10 vol.) and the white precipitate was dried (fraction I; 1·14 g.). The product was non-reducing to Fehling solution. The supernatant solution was concentrated under reduced pressure at room temperature to about 150 ml. and then neutralized by stirring with $BaCO_3$. After filtration, the solution was poured into ethanol (1 l.) to yield a light-brown precipitate (fraction II; yield, 2 g.).

The cation-exchange resin used in the procedure above to deionize the original solution was shaken for several hours with aq. NH_3 soln. (sp.gr. 0·88; 100 ml.). The resin was filtered off and the filtrate concentrated under reduced pressure to a small volume. Addition of excess of ethanol yielded a precipitate (fraction III; yield 1·61 g.). Fraction IV (0·36 g.) was obtained by repeated treatment of the ion-exchange resin with aq. NH_3 soln. The compositions of the fractions are shown in Table 9.

Properties of fraction I. Paper chromatography revealed a single periodate-reacting spot on the starting line (solvent 3). Paper ionophoresis in 25 mM-sodium tetraborate at 3 mA indicated that the material was homogeneous and moved round the cathode (5·5 cm./3 hr.). After hydrolysis, 2-amino-2-deoxyglucose, galactose and mannose were detected. An equivalent weight of 454 was found by titration with 0·01N-NaOH with phenolphthalein as indicator.

The consumption of periodate, under unbuffered conditions and at pH 3·5, is shown in Table 10. During this oxidation no formaldehyde was detected with the Hough *et al.* (1956) technique, but 1 mole of NH_3 was liberated from 770 g. of fraction I. The composition of fraction I before and after periodate oxidation is shown in Table 9.

An attempt was made partially to hydrolyse fraction I under the same conditions as Levene (1941) had used for the preparation of a disaccharide from a similar fraction. Fraction I (0·99 g.) was kept in 10N-HCl (10 ml.) for 45 hr. at 25°. Excess of HCl was removed by allowing the solution to evaporate in a vacuum desiccator over P_4O_{10} and KOH pellets. The residue was extracted successively with hot

Table 10. *Consumption of periodate by fraction I*

Consumption of periodate and the acid produced during oxidation were determined by the methods of Neumüller & Vasseur (1953) and Anderson *et al.* (1955) respectively.

Time of oxidation (hr.)	Consumption of periodate (g. of fraction I/mole of $NaIO_4$)		Acid production (g. of fraction I/mole of acid)
	pH 3·5	Unbuffered	
0·5	293	339	0
1	304	315	1210
3	226	221	808
6	211	205	808
24	163	163	808

methanol and ethanol, and the extracts were evaporated to a solid (0·54 g.), which was dissolved in water (20 ml.). The precipitates obtained by the addition of 4 and 9 vol. of ethanol to this solution were collected (fractions IA and IB respectively; Table 7). The supernatant liquid was concentrated to a syrup (350 mg.), which was shown by paper chromatography to contain amino acids, mono- and oligosaccharides. Fractionation of the mixture on a squat column of acid-washed charcoal (British Drug Houses Ltd.; Andrews *et al.* 1956) yielded, on elution with aq. 5% (v/v) ethanol, a syrup (17 mg.), which gave on paper chromatograms only one spot (R_{Gal} 0·40; solvent 5). This material showed a pink colour with the Elson–Morgan spray reagent (Partridge, 1948) and a purple colour with ninhydrin. Hydrolysis, followed by paper chromatography, revealed the presence of equal amounts of mannose and 2-amino-2-deoxyglucose, but after reduction of this component with potassium borohydride followed by acid hydrolysis only mannose could be detected with *p*-anisidine. The periodate–*p*-anisidine spray revealed an additional polyol (R_{Gal} 1·21; solvent 2), corresponding to 2-amino-2-deoxyglucitol.

Ovalbumin. When similar procedures were applied to ovalbumin no enrichment of the carbohydrate was found. Thus the fraction obtained after treatment with baryta was found to contain 3·7% of mannose and 1·8% of 2-amino-2-deoxyglucose.

Other attempts to isolate the carbohydrate moiety. Holdsworth (1952) cleaved the cell-wall mucoprotein of *Corynebacterium diphtheriae* into carbohydrate and protein by reaction with boiling aqueous picric acid. Investigation revealed that ovomucoid was stable to this treatment.

It was thought that the glycopeptide linkages might be acid-labile. Hence, ovomucoid heated at 80° for 16 hr. at pH 2·0 (maintained by addition of 0·01 N-H_2SO_4) yielded an electrophoretically homogeneous, dialysable fragment which contained mannose, galactose, glucosamine and amino acids; little enrichment of the carbohydrate was evident.

Carbohydrate liberation from ovomucoid did not occur with the following enzymes: pepsin, papain, hyaluronidase (Schering, Berlin), pectinase (Carter and Co. Ltd., Coleford), 19 A.P. (Rohm and Haas Co., Philadelphia, Pa., U.S.A.), bacterase (Norman Evans and Rais, Manchester) and liver enzymes prepared from *Helix pomatia* (Howe & Kabat, 1953).

RESULTS AND DISCUSSION

Ovomucoid

Physical properties. Ovomucoid was prepared by ethanol fractionation of the supernatant liquid resulting from the precipitation of the other egg-white proteins with trichloroacetic acid. This method was preferred to that of Neuberger & Yuill (1940), in which heat coagulation was used to precipitate the other proteins, since, during heating, ovomucoid may be modified with change in the isoelectric point, sedimentation constant and viscosity (Fredericq & Deutsch, 1949) and with partial loss of anti-tryptic activity (Lineweaver & Murray, 1947). Although electrophoresis revealed a single peak at pH 6·9 and I 0·13, spreading of the peak suggested heterogeneity. At low ionic strengths (0·01) and at pH 4·4 at least two peaks were obtained during electrophoresis. Fredericq & Deutsch (1949) and Bier, Terminiello, Duke, Gibbs & Nord (1953) have made similar observations but the fractionation reported by the latter group of authors was probably incomplete since anti-tryptic activity was found in all of their fractions. Evidence of heterogeneity was also obtained by Jutisz, Kaminski & Legault-Démare (1957), who resolved ovomucoid into two components by zone electrophoresis on a column of cellulose; only one of the components possessed anti-tryptic activity. Rhodes, Azari & Feeney (1958) have recently described a promising method for the fractionation and purification of egg-white proteins by ion-exchange chromatography on carboxymethylcellulose. Their ovomucoid fraction had a specific activity similar to that prepared by the trichloroacetic acid method. Ovomucoid showed a weak absorption at 277·5 mμ, no other absorption peak being detected, thus showing the absence of flavoprotein. No further purification of the ovomucoid preparation was attempted in view of difficulties involved in preparing quantities sufficiently large for structural investigations. Ovomucoid appears to be homogeneous by ultracentrifuge-sedimentation analysis (Fredericq & Deutsch, 1949), and ionophoresis of ovomucoid on paper impregnated with borate buffer at pH 9 gave a single band.

Identification of the monosaccharide components. Early workers (Fränkel & Jellinek, 1927; Levene & Mori, 1929; Sørensen & Haugaard, 1933) found D-mannose and 2-amino-2-deoxyglucose. D-Galactose was first characterized by isolation of the tetramethyl ether from a methylated fraction of ovomucoid (Stacey & Woolley, 1942). We were able to characterize D-galactose and D-mannose as the 1-methyl-1-phenylhydrazone and phenylhydrazone derivatives respectively from an acid hydrolysate of ovomucoid. 2-Amino-2-deoxy-D-glucose was

isolated as the hydrochloride from a hydrolysate which had been passed through a charcoal column. The amino sugar is believed to exist as the *N*-acetyl derivative (Stacey & Woolley, 1940). The presence of 2-amino-2-deoxygalactose in ovomucoid (Masamune & Yoshizawa, 1950) was not verified with a sensitive ninhydrin-oxidation method (Ruelius & Girard, 1954).

Quantitative analysis of the carbohydrate. The quantitative analysis of the monosaccharide components of mucoproteins is complicated for various reasons. In general, difficulties arise from the presence of several closely related sugars which can react with amino acids, with loss of carbohydrates, to give products which interfere with the estimations of both amino sugars and other monosaccharides (Immers & Vasseur, 1952; Rondle & Morgan, 1955). It is not surprising that previous investigations of the composition of ovomucoid (Needham, 1927; Karlberg, 1936; Fredericq & Deutsch, 1949; Bier *et al.* 1953; Dixon, 1955; Gottschalk & Ada, 1956) have shown considerable variations. Thus the ratio of 2-amino-2-deoxy-D-glucose:hexose has varied from 1:1 (Dixon, 1955) to 2:1 (Fredericq & Deutsch, 1949).

Cation-exchange resins (H^+ form) have been used with considerable advantage for the hydrolysis of mucoproteins (Glegg & Eidinger, 1954; Dixon, 1955) since sugar–amino acid interactions are minimized by the removal of amino sugar and amino acids on the resin whilst the neutral sugars remain in solution. Resin hydrolysis of ovomucoid was found to be complete in 30 hr. The method appeared to be superior to any other for the quantitative estimation of neutral monosaccharides present in a mucoprotein. 2-Amino-2-deoxy-D-glucose was determined by two independent methods (Belcher *et al.* 1954; Tracey, 1952). With these methods we have found the ratio 2-amino-2-deoxy-D-glucose:D-mannose:D-galactose to be 7·3:3·7:1 (Table 1). The total reducing power (21–23% of hexose; Table 3), as determined by Somogyi's (1945) method, agrees well with the totalled averages (20–21%) of the monosaccharides determined separately.

Rate of hydrolysis of the carbohydrate components. No monosaccharides were liberated on heating an aqueous solution of ovomucoid, adjusted to pH 2·0, at 80°, thus suggesting the absence of furanosyl units.

The liberation of monosaccharides during the stronger acid hydrolysis of ovomucoid was followed since the results could afford useful structural evidence. In the presence of cation-exchange resin (H^+ form), D-galactose was completely liberated within 1 hr., during which time only one-third of the total D-mannose appeared free; the remaining mannose required 48 hr. for complete liberation. The resin-hydrolysis data are thus compatible with the situation in outer positions of the mucoprotein molecule, of all the D-galactopyranosyl units and of about a third of the D-mannopyranosyl units, the remaining D-mannopyranosyl units occupying inner positions.

During acid hydrolysis of 2-acetamido-2-deoxy-D-glucose with 2N-H_2SO_4, the total reducing sugar and amino sugar appeared at similar rates; the hydrolysis was complete in 12 hr. (Table 3). During the acid hydrolysis of galactomannan polysaccharides, which are composed of D-galactopyranosyl end groups in attachment with chains of D-mannopyranosyl units, the galactose also appeared first, followed by mannose (Henderson, Hough & Painter, 1958). Likewise the fucose units present in non-reducing end-group positions in the blood-group mucopolysaccharides A and O were recognized by their preferential liberation during hydrolysis with acid (Kabat & Leskowitz, 1954). De-*N*-acetylation of 2-acetamido-2-deoxy-D-glucopyranosyl units during acidic hydrolysis of the mucoprotein (Foster, Horton & Stacey, 1957) would impede the liberation of these units and also of mannose if it were linked to the reducing group of the amino sugar.

Periodate-oxidation studies. Although a number of mucopolysaccharides have been studied by the periodate-oxidation technique (Jeanloz & Forchielli, 1950, 1951; Aminoff & Morgan, 1951; Holdsworth, 1952), mucoproteins have not been examined in this way. Ovomucoid was oxidized with sodium metaperiodate in unbuffered solution, at pH 6·96 and at pH 3·5, all reactions being carried out in the dark and at room temperature (Table 4). In all three cases there was little difference in the reaction with periodate and consequently it is not complicated by over-oxidation processes involving the progressive removal of units from the reducing end of a chain of monosaccharide units by oxidation through malonaldehyde and formyl ester intermediates (Hough *et al.* 1956, 1957, 1958).

The oxidations occurred in two stages, consisting of an initial rapid uptake of periodate over about 2 hr., followed by a much slower oxidation over about 48 hr. The first stage can probably be accounted for mainly by oxidation of carbohydrate residues, since in general amino acids and peptides are oxidized slowly (Bragg & Hough, 1958). The second stage is undoubtedly due to over-oxidation processes involving oxidation of the carbohydrate and the amino acid residues. Vasseur (1952) found that cystine, cysteine, tyrosine and tryptophan residues of the protein jelly-coat substance of *Echinocardium cylindrica* were attacked by periodate, and Maekawa & Kushibe (1954) observed a similar destruction of amino acids during periodate oxidation of ovalbumin.

The presence of *N*-acetyl groups on the 2-amino-2-deoxy-D-glycosyl residues limits the consumption of periodate to, at most, 1 mole of periodate/amino sugar residue, since oxidation between an acetamido group and an adjacent hydroxyl group would be negligible (Aminoff & Morgan, 1951; Hough & Taha, 1956). The non-reducing end groups of D-galactopyranose and D-mannopyranose would react with 2 moles of periodate with elimination of 1 mole of formic acid. Reliable estimates of formic acid cannot be obtained in the presence of large amounts of protein. Anderson *et al.* (1955) oxidized mixtures of starch and protein and found that, provided that the protein content was less than 23% and not removed from the reaction solution, it had little effect on the determination of formic acid. With ovomucoid, however, the protein content is much higher and is probably responsible for the low yield of formic acid (2–3 moles/mole of ovomucoid).

Since the periodate uptake had suggested that certain monosaccharide units in ovomucoid were not oxidized it was of interest to investigate the composition of the product at the end of the initial rapid reaction. Paper chromatography of a hydrolysate of the oxidized product revealed the presence of D-mannose and 2-amino-2-deoxy-D-glucose but D-galactose was absent. Quantitative determinations showed that 46–48% of the 2-acetamido-2-deoxy-D-glucose residues and about 50% of the mannose residues which were originally present in ovomucoid had remained unoxidized (Table 5). On the structure proposed by Stacey & Woolley (1942) only a third of the mannose units would have been oxidized. The periodate-oxidation data are in agreement with the rate of hydrolysis results and confirm that D-galactopyranosyl and D-mannopyranosyl units occupy terminal positions in the mucoprotein molecule. Verification of this conclusion was sought by methylation of the mucoprotein.

Methylation studies on ovomucoid. Stacey & Woolley (1940, 1942) have, on the basis of methylation studies, described a structure for the carbohydrate moiety of ovomucoid. They isolated 2-acetamido-2-deoxy-3:4:6-tri-*O*-methyl-D-glucose (7 moles), D-mannose (2 moles), 3:4:6-tri-*O*-methyl-D-mannose (1 mole) and 2:3:4:6-tetra-*O*-methyl-D-galactose (1 mole). Since their method involved the use of strong alkali with dimethyl sulphate, we investigated two alternative methods of methylation, namely the action of methyl iodide and sodium upon ovomucoid in liquid ammonia (Freudenberg *et al.* 1938) and the action of methyl iodide on a thallous complex of ovomucoid (Campbell *et al.* 1948). Unfortunately, both methods produced negligible methylation (OMe, $<5\%$) and consequently a slight modification of Stacey & Woolley's (1940) procedure was employed. Since the methylated products contained polypeptidic material it was not possible to ascertain from the total methoxyl content of the product whether or not the carbohydrate component was fully methylated. To ensure that the carbohydrate was fully methylated, the product was then acetylated and treated with silver oxide and methyl iodide but this treatment lowered the methoxyl content of the product. A hydrolysate of the methylated product was fractionated on a charcoal column with fractional elution with ethanol–water mixtures, a procedure which not only separated the methylated sugars, but also freed them from substantial amounts of amino acids. The isolation of 2:3:4:6-tetra-*O*-methyl-D-galactose and 2:3:4:6-tetra-*O*-methyl-D-mannose, characterized as their respective anilides, confirmed the previous suggestions that D-galactose and D-mannose occupy the non-reducing end-group positions in the carbohydrate moiety of ovomucoid. D-Mannose must also occur at other positions in the molecule, in agreement with periodate-oxidation data, since unidentified tri-*O*- and di-*O*-methyl derivatives were detected. The di-*O*-methylmannose gave no formaldehyde either before or after reduction with sodium borohydride and hence it is probably the 2:6-di-*O*-methyl derivative. Methylated derivatives of 2-amino-2-deoxy-D-glucose were not detected, which may have been due to the severity of the methylating conditions or to *N*-methylation of the amino sugar during the reaction with methyl iodide and silver oxide, and subsequent complex formation with silver oxide (Stacey & Woolley, 1942). Bray and James (quoted by Folkes *et al.* 1950) were unable to find any 2-amino-2-deoxyhexose derivatives in a hydrolysate of methylated frog-spawn mucin, a mucopolysaccharide which originally contained 2-amino-2-deoxy-D-glucose and -D-galactose. No free D-mannose was found in our methylated product, in disagreement with the results of Stacey & Woolley (1942).

Attempted enrichment of the carbohydrate moiety of the mucoprotein. Enrichment of the carbohydrate components of the egg-white mucoproteins has been claimed by treatment with barium hydroxide solution at 100° for varying periods of time to hydrolyse the protein (Fränkel & Jellinek, 1927; Levene & Mori, 1929; Suzuki, 1941; Stacey & Woolley, 1940). Fränkel & Jellinek (1927) obtained a product in which the acetamido groups were largely retained, whereas Levene & Mori (1929), who increased the time of heating from 3 to 7 hr., found that complete de-*N*-acetylation of the acetamido sugar had occurred. Determination of the carbohydrate composition of the products formed from ovomucoid by treatment with barium hydroxide in the presence of oxygen-free nitrogen has now revealed that degradation of some of the carbohydrate units had occurred. This result was

not surprising since it is well established that poly- and oligo-hexosaccharides containing 1:4- and 1:3-linkages are rapidly degraded in alkali from the reducing end of the molecule with the formation of saccharinic acids (Kenner, 1955; Kenner & Corbett, 1955). Furthermore, any 2-amino-2-deoxy-D-glucose at the reducing end-group positions would be rearranged and degraded (Stanley, 1953; Knox & Morgan, 1954), although Levvy & McAllen (1959) have found that the amino sugars are more stable than is generally believed.

Fractional precipitation of the alkali-stable products from ovomucoid, followed by passage through a cation-exchange resin, yielded two acidic fractions (I and II; Table 7), which suggested the formation of saccharinic acids from the reducing end of the carbohydrate chain. Two basic fractions (III and IV) were isolated from the cation-exchanger by elution with ammonia solution. In an attempt to isolate disaccharides from fraction I, it was treated with 10N-HCl at room temperature to give fractions IA and IB (Table 7). In no case was a preparation completely devoid of amino acids, although it was significant that the acidic fractions (I, IA, IB) contained markedly increased carbohydrate contents whereas the basic fractions (II, III, IV) did not. Each fraction contained mannose, galactose and 2-amino-2-deoxyglucose, but the composition varied considerably. However, the ratio mannose:2-amino-2-deoxyglucose was found to vary only slightly between 1·0 and 1·3. In every case the galactose content was enriched in comparison with the other monosaccharides, in agreement with its occurrence as non-reducing end groups, which will be unaffected by alkali. Thus the overall effect of alkali on ovomucoid is to cause hydrolysis of the protein and to degrade the carbohydrate, probably by stepwise removal of some alkali-labile 2-amino-2-deoxy-D-glucose and D-mannose units until a branching point is reached (e.g. a $1 \rightarrow 2$ or $1 \rightarrow 6$ linkage), so arresting the degradation process. In this way the D-galactose units and those D-mannose units which occupy non-reducing end-group positions will be preserved.

The acidic fraction I was non-reducing and oxidation with periodate destroyed all of the galactose (Tables 7 and 8), in agreement with galactose end groups. Partial hydrolysis of fraction I, followed by charcoal chromatography, gave in small yield a product which from its behaviour on paper chromatograms appeared to be a disaccharide composed of mannose and 2-amino-2-deoxyglucose. Treatment of the disaccharide with sodium borohydride reduced only the amino sugar component, thus suggesting that mannose is glycosidically linked to 2-amino-2-deoxyglucose. Levene (1941) isolated from ovalbumin a disaccharide with the properties of a 2-amino-2-deoxyglucopyranosylmannose.

Partial acid hydrolysis of ovomucoid followed by fractionation on a squat charcoal column afforded a disaccharide composed only of mannose. Insufficient quantity of this material was obtained for characterization but, since formaldehyde was liberated on periodate oxidation at pH 8, the absence of a $1 \rightarrow 5$ or $1 \rightarrow 6$ linkage was suggested (Hough & Perry, 1956).

Attempts to cleave the protein–carbohydrate bond. The type of bonding between protein and carbohydrate in ovomucoid is unknown but appears to be different from the polar interactions between amino, sulphate and carboxylic acid groups which constitute the major form of bonding in mucopolysaccharide–protein complexes. The available evidence suggests that it is of a covalent nature. That it does not involve highly ionized groups was revealed by potentiometric titration. In a corneal mucopolysaccharide, Woodin (1954) was able to show a protein–carbohydrate bond between sulphuric acid ester and amidine groups by the partial dissociation of the bonds during electrophoresis in the presence of guanidine hydrochloride. Under these conditions ovomucoid gave a single peak in the Tiselius apparatus.

Holdsworth's (1952) picric acid method, treatment with dilute sulphuric acid and incubation with various enzymes failed to yield protein-free carbohydrate fractions.

Ovalbumin

Composition. Previous studies on the electrophoresis of ovalbumin have shown that it is not a single substance, either in the crystalline form or in egg white, and that the mucoprotein occurs in the diphosphate, monophosphate and free forms (Cann, 1949; Durieux & Kaminski, 1956). The diphosphate is usually the major component and the other two components are formed by progressive loss of phosphate (Perlmann, 1950). Crystalline ovalbumin was examined (pH 9; I 0·13) in the Tiselius electrophoresis apparatus, but, although it gave a single peak, this was not symmetrical, which suggested the presence of closely related substances. Steven & Tristram (1958) separated ovalbumin into three components by starch-gel electrophoresis in borate buffer (pH 8·6), but they found only slight differences in electrophoretic mobility.

Ovalbumin has an average molecular weight of 45 000 and contains about 3–4% of carbohydrate, composed of approximately 5 units of D-mannose and 3 units of 2-acetamido-2-deoxy-D-glucose (Levene & Mori, 1929; Sørensen, 1938; Neuberger, 1938; Johansen *et al.* 1958). Examination of an acid hydrolysate of ovalbumin confirmed the presence of D-mannose (2·75%) and 2-amino-2-deoxy-D-glucose (1·4%) in the molecular proportions of 2:1 respectively.

Structural studies. As with ovomucoid the periodate oxidation of ovalbumin was also a two-stage reaction at pH 3·6, but the amount of oxidant consumed at the end of the first stage (1 mole/1180 g. of ovalbumin) was far in excess of that required for the complete oxidation of the carbohydrate content (3·9 %) and thus revealed that extensive oxidation of amino acids was also occurring. Maekawa & Kushibe (1954) found that periodate oxidation completely destroyed cysteine and tryptophan in ovalbumin. Quantitative analysis of the carbohydrate remaining in the product after the initial rapid oxidation revealed that two residues of both mannose and 2-acetamido-2-deoxyglucose were unoxidized. It follows therefore that four mannose units and one 2-acetamido-2-deoxyglucose unit had been destroyed during the oxidation.

Exhaustive methylation of ovalbumin by a similar method to that used for ovomucoid yielded a product in which amino sugar derivatives were not detectable. After fractionation of an hydrolysate of the methylated product on a charcoal column, 2:3:4:6-tetra-*O*-methyl-D-mannose was characterized, thus suggesting that it occurs as non-reducing end groups in the carbohydrate portion of the mucoprotein. Uncharacterized di- and tri-*O*-methyl-D-mannoses were also present. These results are in accord with the periodate-oxidation experiments.

Ovalbumin has been degraded by the use of a variety of proteolytic enzymes to give oligosaccharides and glycopeptides (Cunningham *et al.* 1957; Jevons, 1958; Johansen *et al.* 1958). Examination of the glycopeptides has suggested that one of the carboxyl groups of aspartic acid is directly linked to the carbohydrate, thus affording the first evidence of the carbohydrate–peptide bond.

An attempt was made to enrich the carbohydrate moiety by heating denatured ovalbumin in 10 % (w/v) barium hydroxide solution in the presence of oxygen-free nitrogen (Levene & Mori, 1929), but in agreement with Neuberger (1938) little enrichment was obtained because of the alkaline degradation of the carbohydrate. The product (2 % yield) contained 3·7 % of D-mannose and 1·8 % of 2-amino-2-deoxy-D-glucose.

Partial hydrolysis of ovalbumin by heating at 80° in a dilute solution of trichloroacetic acid for 12 hr., during which time the pH has risen from 1 to 7, was more successful. The water-soluble component (sulphated ash, 50·2 %), which was insoluble in aq. 90 % methanol, contained 4·7 % of mannose and 5·3 % of 2-amino-2-deoxyglucose; considering the high ash content, this result shows that a glycopeptide in which a high proportion of the carbohydrate was present had been released. Since the glycopeptide contained approximately equal amounts of mannose and amino sugar, whereas in ovalbumin the ratio is 2:1 respectively, it would appear that hydrolysis had cloven the carbohydrate moiety of the mucoprotein, leaving a mannose-rich fragment in attachment. A *N*-terminal end-group analysis of the glycopeptide revealed only dinitrophenylalanine.

SUMMARY

1. A semi-micro analytical procedure has been developed for the analysis of the carbohydrates present in mucoproteins. Ovomucoid was found to contain 2-amino-2-deoxy-D-glucose (12·3–12·8 %), D-mannose (6·3 %) and D-galactose (1·7 %), whereas ovalbumin contained D-mannose (2·75 %) and 2-amino-2-deoxy-D-glucose (1·34 %).

2. Specific cleavage of the protein–carbohydrate in ovomucoid and ovalbumin did not occur on treatment with picric acid, barium hydroxide or 0·01N-sulphuric acid.

3. Various carbohydrate-enriched fractions were obtained by the action of barium hydroxide on ovomucoid. It is suggested that in this process the carbohydrate portion of the mucoprotein was also degraded from the reducing end with the loss of some mannose and amino sugar units and consequent enrichment of the galactose component.

4. The partial acid hydrolysis of ovomucoid and ovalbumin was studied. Two disaccharides, a mannobiose and a D-mannosyl-2-amino-2-deoxy-D-glucose, were isolated in very small amounts from partial hydrolysates of ovomucoid. A glycopeptide was isolated from ovalbumin.

5. The action of periodate on ovomucoid and ovalbumin was a two-stage reaction involving oxidation of both carbohydrate and protein.

6. D-Galactose and D-mannose were shown to occur as non-reducing end groups in ovomucoid by using controlled hydrolysis, periodate oxidation and methylation techniques.

7. Methylation of ovomucoid and ovalbumin yielded products in which 2-amino-2-deoxy-D-glucose derivatives could not be detected.

8. D-Mannose was found to occur as non-reducing end groups in the methylated carbohydrate of ovalbumin.

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Characterization of Naturally Occurring Materials which Restore Excitability to Isolated Cerebral Tissues

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Cerebral tissues kept in cold media lose their respiratory and other responses to applied electrical pulses (Marks & McIlwain, 1959). The following properties show that the unresponsive state induced in this way is relatively well defined: (1) several metabolic characteristics of the tissue, in the absence of pulses, were little affected when the metabolic response of the tissue to pulses was depressed; (2) unresponsive states could be induced in the tissue by keeping it under other adverse conditions, but differed from that induced in cold media in several ways, including the type of substances which contributed to restoring response (McIlwain & Gore, 1953; McIlwain, 1956); (3) a variety of agents added to the tissue while it was kept in cold media did not affect the loss of its excitability.

By using as a test system the tissue made inexcitable in cold media, materials which could restore excitability were shown to be present in blood plasma and in the brain. The following properties indicated marked specificity in the action of restoring agents: (1) the active preparations were without appreciable effect on the respiration of untreated cerebral tissues, whether or not pulses were applied to them; (2) the restoring agents had little or no effect on the respiration of the treated tissue in the absence of applied electrical pulses; (3) the effects of the restoring agents were not shared by extracts of muscle or by some 30 known compounds or groups of compounds. An initial exploration of the chemical properties of the active material in blood plasma was described by Marks & McIlwain (1959) and by McIlwain (1959*a*; 1960*a*); it is the further characterization of this material, and of an active substance from the brain, which are now to be described.

During the initial stages of this study, and while the active substances of blood plasma were still uncharacterized, preparations from plasma were used to study the nature of the change which occurred when the tissue was kept in cold media. It was concluded that the unresponsive state so engendered was caused by migration of the tissue's histone. Further evidence regarding this is given in an accompanying paper (Wolfe & McIlwain, 1961). An analogous unresponsive state could be induced in normal cerebral tissues by adding histones and related compounds, and this unresponsive state also has therefore been studied in the present investigation.

EXPERIMENTAL

Tissue metabolism and response to pulses

The cerebral cortex from guinea pigs was used and its response studied in the following experimental arrangements:

Exposure at 0°. Tissues were prepared and exposed in cold media as specified by Marks & McIlwain (1959), with conditions *C* in which cortical slices were kept at 0° for 5 hr. in glucose-containing media buffered with 2-amino-2-hydroxymethylpropane-1:3-diol (tris). After being kept at 0° the tissue specimens (inner slices with two cut surfaces, fresh wt. about 45 mg.) were mounted in silver-grid electrodes and placed in vessels *E* (Ayres & McIlwain, 1953) containing 3·5 ml. of glucose–tris medium. In some of the vessels the medium received the substances which were being examined as agents restoring excitability. Respiratory rate was measured during 40–45 min. at 37·5°, after which alternating condenser pulses of peak potential 10 v and 0·4 msec. time-constant were applied at 100/sec. and measurement of respiration was continued for a further 40–60 min. At the end of an experiment the electrodes with their tissues were removed promptly from the vessels.

Inhibition by proteins. The unresponsive state induced by basic proteins was examined as specified by McIlwain (1959*a*): immediately after weighing, the tissue samples were placed in the electrodes of the apparatus described above, immersed in glycylglycine-buffered media, and the respiration was measured at 37·5° before and during the application of electrical pulses. The following three types of experiment were involved:

(*a*) The inhibitory potencies of different proteins and peptides were compared by including them in a range of concentrations in the experimental media into which the slices were placed.

(*b*) The ability of added substances to restore excitability to tissue exposed to basic proteins was examined under the conditions of McIlwain (1959*a*, Tables 4 and 5). This involved incubating tissue specimens in electrodes in a series of vessels, some with and some without the basic proteins, for 15 min. The vessels were then taken from the thermostat and from their manometers, and the media removed from the vessels with Pasteur pipettes. The tissues remained in their electrodes in the vessels during this procedure, and the tissues, electrodes and vessels were further washed with 1 ml. of medium which in all the vessels was without

inhibitor. Medium alone, or media with the substances being examined as agents restoring the excitability, were then pipetted into the vessels. The vessels were replaced on their manometers, re-equilibrated with oxygen, and respiration was measured before and during the application of pulses as above.

(*c*) For examining the capabilities of added substances in preventing the establishment of an inhibitory condition in the presence of basic proteins, media containing the protein were added to a series of manometric vessels. To some of the vessels were then added the substances being examined as antagonists. About 15 min. later, tissues in their electrodes were placed in the vessels and the experiments completed as above. In a few instances (Table 4) application of electrical pulses was replaced by the addition of 0·15 ml. of M-KCl from the side arm of manometric vessels, to 3·5 ml. of medium.

Control experiments. The following experiments were carried out to ensure that the changes in gas pressure measured manometrically were due to tissue respiration. Pulses of the parameters described above were applied to vessels containing electrodes, media and the added compounds under examination, but no tissue. No progressive pressure changes were observed; these experiments included the gangliosides (0·4 mg./ml.) to control the experiments of Tables 2 to 4, and gangliosides (0·4 mg./ml.) plus protamine (0·15 mg./ml.) to control those of Tables 3 and 4. Also, as is noted in Table 3, gangliosides were examined in the presence of normal tissue (i.e. not kept at 0°) and were not found to increase its respiratory response to pulses.

Materials and analyses

Ox-blood plasma fractions and cerebral extracts were those described by Marks & McIlwain (1959). Basic proteins included those described by McIlwain (1959*a*), the protamine being clupein sulphate (L. Light and Co. Ltd.) unless otherwise stated. Analysis of the specimen was as follows (Weiler and Strauss, Oxford): total N, 24·6; total S, 5·78%. These values are very close to those of Carroll, Callanan & Saroff (1959) for purified salmine and, as the protamines have no sulphur-containing amino acids, the values imply an equivalent weight of 277 for the specimen used (cf. Table 6). For a freshly made specimen of calf-thymus histone sulphate, I am indebted to Dr L. S. Wolfe; it gave: total N, 13·85; total S, 4·31%. On a similar basis (see Crampton, Stein & Moore, 1957) this implies an equiv. wt. of 371. The specimen of poly-L-lysine hydrochloride, degree of polymerization approx. 30, was from Mann Research Laboratories Inc., New York. A specimen of the sialomucopolysaccharide, isolated from human ovarian-cyst fluid by Pusztai & Morgan (1960), was kindly given by Professor W. T. J. Morgan.

Gangliosides. Two preparations (I) of ox-brain gangliosides were made largely according to Folch, Lees & Sloane-Stanley (1957), the choice of some conditions being that of Long & Staples (1959). Two ox brains were freshly obtained from a slaughter house and cerebral cortex (50 g.) was taken with relatively little admixture with white matter, by snipping off portions of about 0·4 g. with scissors. This was blended in a top-drive macerator with the chloroform–methanol mixture (950 ml.) for 2 min. and the suspension filtered by gravity through fluted Whatman no. 43 papers. The filtrate was distributed among six 200 ml. centrifuge bottles and one-sixth of its vol. of 0·1 M-KCl added to each. The bottles were stoppered and shaken well, so that an emulsion was maintained for 15 min., and were then centrifuged at 1500 *g* for 20 min. The upper layers were collected by suction for the main ganglioside preparations. Two subsequent extractions (*c*) of the lower layers were made but the materials obtained have not been used in the present study. Each upper phase was dialysed in Visking tubing (diam. 1 in.) tied to form sacs which were immersed in five successive batches of 16 l. of distilled water at 0–2°; they remained with each batch of water for approx. 12 hr. The water was contained in cylinders 52 cm. high and agitated with a Vibromix stirrer (Shandon Scientific Co. Ltd., London; from A. G. für Chemie-Apparatebau, Zürich), which is very effective in aiding dialysis as material inside the sacs is also agitated by the 50 cyc./sec. vibration. After dialysis the sac contents were evaporated at a few mm. Hg (bath temp. 60°, vapour temp. 20°) to about 8 ml., and the slightly yellowish solution was washed into a Visking sac (diam. $\frac{1}{2}$ in.) and then dialysed as before for 16 hr. The sac contents were filtered into a crystallizing dish and this was placed above $CaCl_2$ in a desiccator, which was evacuated. Fine white strands separated in about half an hour, before the solution had evaporated, and after some hours were left as a light white fibrous solid, yield 93 mg. The two extractions (*c*), when treated in the same way, yielded yellow scales 0·1 and 0·03 of the weight of the main batch.

A further ganglioside preparation (II) was given by Dr C. Long and was the 'partially purified ox-brain strandin' of Long & Staples (1959), having been prepared by Dr G. H. Sloane-Stanley. For a preparation (III) made according to Folch, Arsove & Meath's (1951) procedure for strandin, I am indebted to Dr S. Balakrishnan. A preparation (IV), purified by chromatography on silicic acid according to W. E. van Heyningen & P. M. Miller (unpublished work) was given by Dr W. E. van Heyningen.

Neuraminic acid content. Determinations were carried out with Bial's orcinol–$FeCl_3$–HCl reagent; for the synthetic *N*-acetylneuraminic acid (Carroll & Cornforth, 1960) used as standard, I am indebted to Miss Patricia Carroll. In plasma fractions the reagent was used as described by Böhm, Dauber & Baumeister (1954), with samples usually of 5 mg. of the fractions. In cerebral preparations the method of Long & Staples (1959) was used: a sample equivalent to 200 mg. of brain was divided into two parts, placed in 10 ml. tubes in a water bath at 80–100° and evaporated to dryness in a stream of N_2. To each tube, after cooling, was added 1 ml. of 4 N-HCl and one tube was heated at 100° for 30 min. and cooled. To each, 1 ml. of Bial's reagent prepared in 4 N-HCl was added, and both tubes were heated for 15 min. at 100°. Extraction with pentan-1-ol, photometric reading and preparation of standards (10–60 μg. of *N*-acetylneuraminic acid) followed the description of Long & Staples (1959).

The ganglioside preparations showed the following content of neuraminic acid derivatives, expressed as the *N*-acetyl compound: I, main batches 24 and 26·5%; II, 22%; III, 10%; IV, 28·5% (quoted by Dr van Heyningen: 0·928 μmole/mg. or 28·7%; and 0·308 μmole of *N*-acetylgalactosamine/mg.). Similarly expressed, the sialomucopolysaccharide contained 17% of neuraminic acid derivatives. Their purification has not been attempted in the present study, but the following data are relevant. The ganglioside preparations of Svennerholm (1956) and of Rosenberg & Chargaff (1958) contained neuraminic acid

equivalent to about 26% of the *N*-acetyl derivative. Meltzer (1958), after extensive fractionation, obtained material containing 33% of 'sialic acid' (the term is a generic one for the various acyl derivatives of neuraminic acid). Svennerholm (1956) obtained the preparation quoted above from cerebral tissues dehydrated with acetone before extracting with non-aqueous solvents; when, as in the present preparation I, extraction was of fresh brain, the product yielded amino acids on hydrolysis. Le Baron & Folch (1957) obtained a similar preparation which yielded a high proportion of arginine among the amino acids obtained on hydrolysis; the formation of complexes, reported below, may be relevant to this result.

Precipitation reactions

The data of Table 5 were obtained by mixing at room temperature (about 16°) the basic proteins and the neuraminic acid derivatives, each dissolved in one of the media already quoted or in 33 mM-glycylglycine brought to pH 7·4 by NaOH. Mixtures were made in volumes of 0·2–0·3 ml. and at dilution intervals of approx. 30%, and they were observed at intervals for about 1 hr. Picric acid was used as a reagent as a saturated aqueous solution of 1·4 mg./ml. or 6·1 mM, and sodium fluorescein at a concentration of 10 mg./ml. or 26·4 mM. They were added to give concentrations in the solutions being tested of 0·6 and 1·66 mM, and the solutions were observed at intervals for some hours. With protamine only, at about 100 μg./ml., picric acid yielded an immediate cloudiness, becoming a flocculent brown precipitate in a few minutes. With histone at a concentration of about 300 μg./ml., this occurred rather more slowly. Fluorescein gave bulkier, darker precipitates with both. Neither picric acid nor sodium fluorescein appeared to interact with the gangliosides or with the sialomucopolysaccharide.

RESULTS

Properties of blood-plasma fractions

Blood plasma and serum from ox and man were previously found of similar activity in restoring excitability to cerebral tissues which had been kept in media at 0°; the active material was non-dialysable, was precipitated by acetone or by ammonium sulphate, and was partly inactivated on heating (Marks & McIlwain, 1959). In the separation of ox plasma with ethanol at defined pH, according to Cohn *et al.* (1946), fractions IV-1 and IV-4 were found markedly more active than others in restoring excitability. A number of qualitative tests have now been applied to these and to the less active fractions. Molisch's reaction for carbohydrates (α-naphthol and a lower layer of conc. sulphuric acid) yielded with the active fractions not only the purple of the test, but also a distinct and intense brown layer above the purple one. A similar, extraneous brown colour was also given in the orcinol–sulphuric acid reaction for protein-bound hexose, carried out according to Winzler (1955), and the brown colour in both tests was then found to be given by the addition of sulphuric acid alone. It was given to a much lesser degree, if at all, by similar quantities of the other plasma fractions. At this point the 'browning reaction' of strandin was recollected. This reaction, observed by Folch *et al.* (1951), was later shown to be due to neuraminic acid derivatives (Rosenberg & Chargaff, 1958). Neuraminic acid derivatives occur in blood plasma in certain of the mucoproteins, and fraction IV-4 was found by Surgenor, Strong, Taylor, Gordon & Gibson (1949) to be rich in mucoproteins, though the type of mucoprotein was not then specified.

These observations prompted the determination of neuraminic acid derivatives in all the plasma fractions previously tested in restoring tissue excitability. Markedly higher contents of neuraminic acid derivatives were found in fractions IV-1 and IV-4 (Table 1). The fractions are precipitated in the order

Table 1. *N-Acetylneuraminic acid of ox-plasma fractions*

The fractions (Marks & McIlwain, 1959) were prepared by the method of Cohn *et al.* (1946), whose enumeration is followed. Determinations were according to Böhm, Dauber & Baumeister (1954) and the values quoted are the average of duplicate or triplicate results agreeing within 5%.

Fraction	*N*-Acetylneuraminic acid (mg./g. dry wt.)
I	4·0
II	2·4
III-1	6·6
III-2	6·8
IV-1	19
IV-4, batch C 183	23·2
IV-4, batch BP 45	23·4
V	2·4
Albumin, crystalline	< 0·02

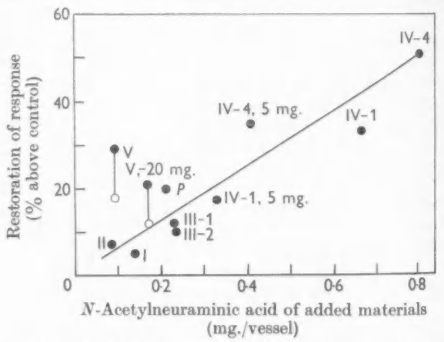

Fig. 1. Restoration of response by blood-plasma fractions in relation to their content of *N*-acetylneuraminic acid. Response is the respiratory response to electrical pulses of a 45 mg. slice of guinea-pig cerebral cortex in 3·5 ml. of fluid, determined as described in the Experimental section. *N*-Acetylneuraminic acid is the content (see Table 1) of material added to the 3·5 mg. of fluid, the additions being of 10 mg. dry wt. of material/ml. of fluid, except when indicated as being 5 or 20 mg./ml. *P*, Plasma; I–V, fractions of Table 1.

listed in Table 1, which thus shows a zone within which the greater part of the combined neuraminic acid is precipitated. This coincided with the precipitation of the greater part of the material most active in restoring tissue excitability. In Fig. 1 the two properties are compared in eight fractions. The correlation is evident; it is also apparent that fraction V forms an exception in having an ability to restore tissue excitability which is disproportionately large for its neuraminic acid content. Here the properties of serum albumin are relevant. A crystalline specimen of the albumin was found (Marks & McIlwain, 1959) to have a limited ability to restore excitability to tissues kept in media at 0°. In quantities between 5 and 60 mg./ml. of medium the specimen was shown to increase respiratory response by 10–17%, and no more; though 5 and 10 mg./ml. gave values of 11 and 12%, 60 mg./ml. gave only 10%. The albumin specimen has now been found to be almost completely free of neuraminic acid derivatives (Table 1). Albumin constitutes the greater part of fraction V (Cohn *et al.* 1946) and it therefore appears reasonable, in appraising the contribution of neuraminic acid derivatives to the properties of fraction V, to allow for the properties of the albumin. This has been done in the two open circles of Fig. 1, when the correlation between restoring ability and neuraminic acid content becomes closer.

Ganglioside preparations and a sialomucopolysaccharide

The major neuraminic acid-containing substances of the brain are the gangliosides; their constituents are not yet completely defined (see Experimental section) but include also galactosamine, glucose, galactose and ceramides (sphingosine-fatty acid amides). Three ganglioside preparations, made by different methods and described above, have been examined for their ability to restore excitability to cerebral tissues kept at 0°. All were active (Table 2). Maximal restoration was obtained with 0·15 mg. (/ml.) of a preparation which corresponded to an addition of 36 μg. (/ml.) of combined *N*-acetylneuraminic acid. Comparison with the ability of the serum fractions to restore excitability (Fig. 1) shows that when the activities of the two categories of material are expressed in terms of their neuraminic acid content, the ganglioside preparations are markedly more potent. *N*-Acetylneuraminic acid itself (0·5 mg./ml.) had little or no ability to restore excitability.

A specimen of a sialomucopolysaccharide, kindly given by Professor W. T. J. Morgan, has, however, shown activity. The compound (Pusztai & Morgan, 1960) was isolated from ovarian-cyst fluid, and contains, in addition to a sialic acid, hexosamine, reducing sugars and fucose. At a concentration of 1 mg./ml. it showed distinct but limited ability to restore excitability to the tissue kept at 0°. Its activity (Table 2) was similar, in terms of neuraminic acid content, to that of the blood-plasma fractions.

Inhibition by basic proteins and a polypeptide; restoration and antagonism by the gangliosides

Histone and protamine preparations at 2–10 μM were previously shown to inhibit metabolic responses by cerebral tissue to applied electrical pulses; excitability was restored to the tissue by further addition of plasma fraction IV-4 (McIlwain, 1959*a*). Results are now reported (Fig. 2) with a laboratory preparation of calf-thymus histone, for which I am indebted to Dr L. S. Wolfe, and with a synthetic polypeptide. The freshly prepared histone was inhibitory in concentrations similar to those at which the previous specimen acted; neither of these

Table 2. *Restoration of response by materials added to tissue kept at* 0°

After exposure at 0° (see Experimental section), slices (45 mg.) were placed in electrodes in manometric vessels containing the added substances in 3·5 ml. of medium. Respiratory rate was measured for 40–45 min., after which pulses were applied for 40–45 min. and the measurement was continued.

Substance added	Weight added (mg./ml. of medium)	Respiratory rate (μmoles of O_2/g./hr.) Without pulses	With pulses	Increase in respiratory rate with pulses (%)
None	—	47±3 (6)	59±5 (4)	25±6 (4)
Plasma fraction IV-4	5	47 (3)	78 (3)	66
Ganglioside preparation I	0·4	49 (2)	86 (2)	75
	0·15	51±4 (4)	92±6 (4)	80±9 (4)
	0·06	48 (2)	73 (2)	52
Ganglioside preparation II	0·4	49 (2)	87 (2)	77
Ganglioside preparation III	0·8	47 (2)	89 (2)	89
Ganglioside preparation IV	0·4	48 (3)	90 (3)	88
Sialomucopolysaccharide	1·0	46±3 (4)	71±5 (4)	53±8 (4)
	0·3	46 (2)	65 (2)	41

preparations nor the protamine has been examined and purified by methods appropriate to large molecules, which have shown that histone preparations contain several distinct basic proteins (Luck, Rasmussen, Satake & Tsvetikov, 1958). The synthetic poly-L-lysine was, however, of similar activity to the protamine, and it would seem that the inhibition being investigated is a fairly general property of such basic polypeptides.

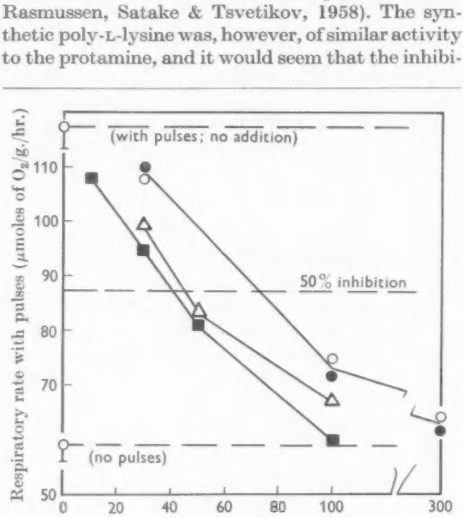

Fig. 2. Depression of respiratory response to pulses by the sulphates of calf-thymus histone (two specimens: ○ and ●), the protamines clupein and salmine (■) and by poly-L-lysine hydrochloride (△; mol.wt. approx. 5000). The substances were present in 3·5 ml. of media to which tissues (approx. 45 mg.) were added. Respiratory rate was measured first without pulses, and was little affected by the additions; this value is given by the lower broken line, with S.D. indicated on the ordinate. Pulses of 10 v peak potential and time constant 0·4 msec. were applied at 100/sec. during a second period, giving the rates recorded. The rate with pulses but without added substances is given by the upper broken line, again with S.D.

The gangliosides have been examined as agents which might restore excitability to cerebral tissues whose respiratory response was inhibited by protamine. Inhibition was first established by incubating tissue samples in media containing 150 μg. of protamine/ml., which was shown in other experiments to prevent completely the response to electrical pulses. The medium was then removed and the tissues, vessels and electrodes were washed with protamine-free media. In some vessels of an experiment, incubation was then continued in fresh, protamine-free solution; the tissue in these vessels remained inexcitable (Table 3). To other vessels ganglioside preparations were added, and in these response occurred. A concentration of 0·14 mg./ml. gave nearly maximal restoration of response.

The gangliosides have been investigated also as agents which might prevent the action of the basic proteins (Table 4). In these experiments, solutions of gangliosides and protamine were made in the usual metabolic media, and normal cerebral tissues subsequently placed in the solutions. Ganglioside preparations were then capable of preventing the inhibition usually caused by the protamine. The quantities of material involved were similar to those of the experiments of the preceding paragraph. In addition, the basic proteins inhibit the normal action of 50 mM-potassium salts on cerebral tissues (McIlwain, 1959*a*); a ganglioside preparation prevented this action also of the protamine (Table 4).

Complex-formation between basic proteins and neuraminic acid-containing compounds of large molecular weight

The fractions of blood-plasma proteins that restored excitability to cerebral tissues kept in media at 0° formed precipitates with histone and prot-

Table 3. *Ganglioside preparations restoring excitability to protamine-treated tissues*

Slices of guinea-pig cerebral cortex were incubated in electrodes in media with or without added protamine sulphate (0·15 mg./ml. when added; see Experimental section) for 15 min., after which these media were removed and replaced by others without protamine but containing gangliosides when indicated. Respiratory rates were measured during two successive periods of 45 min., during the second, pulses of the characteristics quoted in Fig. 2 were applied. Values are the averages of two or three observations which agreed to within 5%, except when followed by S.D. and number of observations.

Additional substances present		Respiratory rate during successive periods (μmoles of O_2/g./hr.)	
During preincubation	During respiratory measurements (mg./ml.)	Without pulses	With pulses
None	None	57±3 (5)	108±6 (5)
None	Gangliosides II, 0·43	59	104
Protamine	None	56±2 (4)	58±5 (4)
Protamine	Gangliosides II, 0·43	60	91
Protamine	Gangliosides I, 0·57	54	97
Protamine	Gangliosides I, 0·14	56	88

amine preparations (McIlwain, 1959*a*). The sialomucopolysaccharide also has now been found to exhibit this property in high dilution (Table 5). Visible precipitation was given in neutral solutions at 60 μg./ml., with the protamine or histone at 100 or 300 μg./ml. These are the concentrations of the basic proteins about minimal for maximum inhibition under the conditions of the preceding experiments. Precipitation increased with increase in concentration of the sialomucopolysaccharide to about 200 μg./ml., but then diminished, and at the higher concentration of Table 5 precipitation did not occur.

In similar experiments ganglioside preparations yielded either no precipitate or only slight precipitates, much less than the maximum obtained with the sialomucopolysaccharide, though concentrations of gangliosides between 10 and 1000 μg./ml. were examined. Two types of ganglioside preparations were used (see Experimental section), one made by a procedure yielding peptide-containing preparations and the other peptide-free; they behaved similarly towards the proteins. Interaction between the gangliosides and the proteins was, however, shown by the addition of further reagents. Picric acid, known to form insoluble salts with protamines and histones (Cohnheim, 1900), and used in the previous studies (McIlwain, 1959*a*), gave an evident precipitate with the proteins (50 μg./ml.) in the buffered media of the present experiments; fluorescein gave a bulkier and darker precipitate (see Experimental section). When, however, small quantities of the gangliosides were present with the protamine or histone, precipitation with picric acid or fluorescein was prevented. The concentrations involved are shown in Table 5.

Moreover, when the sialomucopolysaccharide was added to either of the basic proteins in a quantity sufficient first to form a precipitate and then to

Table 4. *Ganglioside preparations preventing inhibition by protamine*

Slices of guinea-pig cerebral cortex were placed in glycylglycine media in manometric vessels. The media already contained the additional substances of the Table (weights of protamine refer to the sulphate) before the tissues were added. In Expts. A, the tissues were in electrodes; in Expts. B they were floating freely. After 40–50 min. incubation, during which respiration was measured, electrical pulses (as in Fig. 2) were applied to the tissues in electrodes, and additional M-KCl (making the media 50 mM in K^+) tipped from side arms to the other tissues; respiratory measurements were continued for a further 40–50 min. Results are quoted as mean values from the number of observations in parentheses (with standard deviation) or from two to three observations.

Group of expts.	Additional substances present (mg./ml.)	Respiratory rate (μmoles of O_2/g./hr.) 1st period	2nd period
A, B	None	64±3 (6)	131±6 (6)
A	Protamine, 0·11	63±3 (4)	79±5 (4)
A	Protamine, 0·11; ganglioside I, 0·43	63±3 (4)	131±4 (4)
A	Protamine, 0·11; ganglioside I, 0·14	65	108
A	Protamine, 0·11; ganglioside II, 0·43	62	130
B	Protamine, 0·05	65	89
B	Protamine, 0·10	64	69
B	Protamine, 0·10; ganglioside I, 0·29	63	115

Table 5. *Precipitation reactions of basic proteins with a mucopolysaccharide and with ganglioside preparations*

To a series of tubes containing the protein sulphates dissolved in 33 mM-glycylglycine or in tris-buffered media were added varied quantities of the other components in the same media. About 1 hr. after making these mixtures the fluorescein or picric acid solutions were added (see Experimental section). This caused marked precipitation with the basic proteins alone; the other components either (*a*) lessened the extent of these precipitates or (*b*) completely or nearly completely prevented them.

Protein sulphate (μg./ml.)	Other component	Concentration (μg./ml.) of other component causing just noticeable ppt.	maximal ppt.	redissolving of ppt.	with fluorescein or picric acid: (*a*) decreased ppt.	(*b*) little or no ppt.
Protamine, 120	Sialomucopolysaccharide	60	230	720	230	720
Protamine, 120	Ganglioside IV	Little or no precipitate			55	300
Histone, 300	Sialomucopolysaccharide	60	170	350	120	350
Histone, 300	Ganglioside IV	Little or no precipitate			95	300

begin to dissolve it, the basic protein was again protected from precipitation by picric acid or fluorescein. It is therefore suggested that the ganglioside preparations form soluble and fairly firmly bound complexes with the protamines and histones. These interactions are exhibited under the conditions in which their effects on tissue excitability are shown, and in concentrations of 50–300 μg./ml.

By using the precipitation reactions in the same semi-quantitative way, combination between the basic proteins and a constituent of cerebral tissue has been shown to occur during metabolic experiments. After incubating slices of approx. 50 mg. under the conditions of Tables 3 or 4 with protamine (75 or 100 μg./ml.), the slices were removed and fluorescein was added to portions of the medium. The reaction given was markedly diminished by the tissue, and after incubation corresponded to only about one-third of its initial value. When protamine (150 μg./ml.) was added, the residual reaction corresponded to about half the protamine added. This suggests an interaction with about 4 μg. of protamine for each milligram of tissue. All these concentrations of protamine gave complete or almost complete inhibition of response. Because of the finding, which has been previously described, that gangliosides in solution can prevent reaction between protamine and fluorescein, these observations do not decide whether combination occurs at the tissue or in solution, and experiments are in progress to determine this.

DISCUSSION

Keeping cerebral tissues in cold media was chosen for study as being one of the mildest of procedures causing loss of excitability. It does, however, diminish the contribution of metabolically derived energy to the maintenance of structure and of concentration gradients, and possibly in this way permits the interaction of cell constituents which themselves possess mutual affinity. The gangliosides and histones have been shown to have great mutual affinity. They are large molecules, the gangliosides with some 160 acidic groups per molecule and the basic proteins with 20–30 basic groups per molecule; and, as indicated in Table 5, they form complexes in high dilution in the neutral aqueous solutions in which they affect tissue excitability. The simplest interpretation of the properties of these two components, as exhibited after isolation and separate addition to cerebral tissues, is to suppose that the histones act as inhibitors through their combination with tissue gangliosides, and consequently that the gangliosides are normally involved in tissue excitability.

A wider survey of tissue anions is, however, needed before this interpretation can be regarded as established. In addition to gangliosides, the brain contains other neuraminic acid derivatives (Svennerholm, 1956; Brante, 1959). It also contains other acidic substances of large molecular weight. A recent appraisal of ion balance in the brain as a whole (McIlwain, 1959*b*) indicated that, even including recently recognized constituents such as *N*-acetylaspartic acid, some 80 μequiv. of anions/g. of tissue remained to be accounted for by materials such as lipids or proteins. Much of this material, to judge by the properties of cerebral extracts as agents restoring tissue excitability, is not involved in the present phenomena, but certain constituents including the sulphatides are to be noted. The sulphatides (see McIlwain, 1959*b*) are, however, present at greater concentration in white matter than in grey, and a substance concerned with tissue excitability in the present sense would be expected to be distributed in favour of grey matter. The gangliosides are so distributed and in addition occur almost exclusively in neural rather than in the other animal tissues examined (Long & Staples, 1959).

Interacting quantities of gangliosides and basic proteins

Data on the substances examined in the present study are assembled in Table 6. The specimens of gangliosides and basic proteins which have been used are naturally occurring mixtures rather than pure substances; this is also true for the specimens to which many of the data refer, and is to be borne in mind in the following appraisal.

(1) When protamine and poly-L-lysine inhibited the tissue's response by 50 %, they are seen to have been added in molar amounts which are 30–50 % of the molar quantity of *N*-acetylneuraminic acid present in the gangliosides of the tissue on which they acted. Moreover, this quantity of protamine (about 0·7 μmole/g. of tissue) was shown by the experiments above to combine with the tissue or with a tissue constituent, concomitantly with the inhibition of tissue excitability.

(2) When (see Table 5) gangliosides protected the protamine of the solutions of Table 5 from precipitation by fluorescein or picric acid, a quantity of ganglioside containing (see Table 6) about 2 equiv. of *N*-acetylneuraminic acid per mole of protamine partially prevented precipitation; about 8 equiv. were needed to prevent precipitation completely.

(3) Gangliosides, as agents restoring response, acted in quantities within the range to be anticipated from the values of (1) and (2): 50 % restoration was given by solutions containing about 2·5 times the quantity of gangliosides contained in the tissue under test.

(4) The poly-L-lysine of molecular weight similar to the protamine, but with more basic groups, was

Table 6. *Quantities of materials whose interaction conditions response to pulses*

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Substance examined			Amount* of substance (*A*) involved in (*C*)		Interacting quantities derived from (*B*) and (*D*)	
(*A*)	(*B*) Mol.wt. or equiv.wt.	(*C*) Property of substance *A* which is measured	Concentration in solution (μg./ml.)	(*D*) Expressed in relation to tissue wt. (μg./g. fresh wt.)	(μmoles/g. of tissue)	(μequiv./g. of tissue)
Protamine sulphate	Mol.wt. 5000† Equiv.wt. 270 or 830†	50% inhibition of response	40	3100	0·62	11·5; 3·7
Poly-L-lysine hydrochloride	Mol.wt. 4950‡ Equiv.wt. 166‡	50% inhibition of response	50	3900	0·79	23·5
Histone sulphate	Mol.wt $1–2 \times 10^4$§ Equiv.wt. 371**	50% inhibition of response	70	5450	0·3–0·5	14·7
N-Acetylneuraminic acid of gangliosides	Mol.wt. 309	Quantity in cerebral cortex‖	—	525	1·7	—
Gangliosides	Mol.wt. 2×10^6¶ Equiv.wt. 1200¶	50% restoration of response	70	5450	0·027	4·5

* The amounts of substances are derived from the data of Tables 2–4 and Fig. 2.
† Callanan, Carroll & Mitchell, 1957; Carroll, Callanan & Saroff, 1959. Salmine has 18 to 19 basic groups, but 6 strongly basic sites.
‡ Wetlanjer & Stahmann, 1953; Katchalski & Sela, 1958; the equivalent weight is quoted on the assumption that all basic groups are effective.
§ Crampton, Stein & Moore, 1957.
‖ Long & Staples, 1959.
¶ Folch, Arsove & Meath, 1951; Rosenberg & Chargaff, 1958.
** See Experimental section.

active in approximately the same quantity by weight. A greater weight but rather smaller molar quantity of histone was effective.

Relationship of the basic proteins to the tissue

To understand these interactions in detail is likely to require more specific knowledge of the ionization and affinities of the soluble reactants, and also of chemical and geometrical factors concerned in localization of the relevant acidic groups in the tissue. As well as the results of the accompanying paper (Wolfe & McIlwain, 1961), two groups of observations with erythrocytes appear apposite. The main acidic groups which condition the surface charge of these cells are likely to be neuraminic acid derivatives, since they are removed by neuraminidases (Pye, 1955; Gottschalk, 1958; Klenk, 1958). Combination of lysine polymers (including that used in the present study) with erythrocytes has been examined by Katchalsky, Danon, Nevo & de Vries (1959), whose evidence suggested that the molecules of the lysine polymers are fully extended in aqueous solutions, through mutual repulsion of the many positive charges of the molecules. The polymers appeared to become attached to acidic groups of the cell surface by basic groups near one end of the polymer, and to act as agglutinating agents through attachment at the two ends to the surface of two erythrocytes, which were then observed electron-microscopically to be separated by bridges of material about the length of the molecules of the lysine polymers used. In relation to the acidic groups of the cells, the polylysine is presumably then acting as a monobasic or dibasic substance in spite of its many positive charges, a situation implied by some of the interacting ratios observed in cerebral tissues and discussed above.

Evidence has been given by van Heyningen (1959) suggesting that gangliosides may constitute the site of attachment of tetanus toxin to neural tissues. van Heyningen (1959) emphasizes the specificity of the interaction of the gangliosides with the toxin rather than with other proteins. The toxin is indeed effective in minute amounts. However, it is feasible that a firmness of attachment exhibited by the toxin (which is not strongly basic) on account of specific structural relationships to the gangliosides, may be paralleled by that conferred by the multiple positive and negative charges which are the probable basis of the present interactions. These are not dependent on details of protein structure, for a protamine, a histone and an amino acid polymer are all capable of acting as the basic component. Whether the gangliosides may exhibit a special relationship to the cerebral histones is part of an investigation of specificity now in progress. That the cerebral histones, after migration from the nucleus, can condition tissue excitability may be

adventitious but may carry greater significance. It is known that nuclear size and composition in neurons undergo changes according to the level of neural activity; in other types of cell the nuclear proteins undergo changes during cell division. The possible modes by which histones, in their new site, may condition excitability will be realized from the preceding discussion to be manifold; inhibition by protamine is affected by calcium-ion concentration and is accompanied by change in tissue fluids (McIlwain, 1960*b* and unpublished work).

SUMMARY

1. Cerebral tissues kept in cold media do not give their normal respiratory response to pulses when subsequently brought to 37°. Response was restored by including certain fractions of blood-plasma proteins in the media during incubation. The potency of different plasma fractions in restoring response was correlated with their content of neuraminic acid derivatives.

2. Other substances of large molecular weight and containing neuraminic acid also restored the tissue's response: a sialomucopolysaccharide and preparations of gangliosides from the brain which were active at a concentration of 150 μg./ml. of medium.

3. An analogous unresponsive state was induced in normal cerebral tissues by incubation with protamines, histones or a synthetic poly-L-lysine at a concentration of 30–150 μg./ml. Response here also, after inhibition by protamine, was restored by gangliosides. Prior mixture of an amount of protamine normally inhibitory, with a little more than its own weight of gangliosides, prevented the protamine from exerting its inhibition.

4. Concentrations of gangliosides which restored response to the tissue kept cold or with protamine, when added to protamine without tissue, prevented the protamine being precipitated by picric acid or fluorescein. This was attributed to complex formation between gangliosides and protamine; similar phenomena were shown with inhibitory concentrations of histone, and also between the sialomucopolysaccharide and the basic proteins.

5. The quantities of the basic proteins and gangliosides which interact in conditioning the response of a tissue specimen are compatible with the proteins inhibiting by combination with the gangliosides of the specimen. The possibility is discussed of such a mechanism operating when response is lost on keeping the tissue in cold media or with protamine.

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Migration of Histones from the Nuclei of Isolated Cerebral Tissues kept in Cold Media

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Slices of guinea-pig cerebral cortex, kept in buffered glucose media at 0° for 5–17 hr., lose their normal respiratory response to applied electrical pulses. This response can be restored to a considerable extent by Cohn fraction IV-4 of ox plasma (Marks & McIlwain, 1959). Dialysed acid or sodium chloride–ethanol extracts of whole brain and of isolated nuclei also inhibit the isolated tissue's response to electrical stimulation, without affecting normal respiration in the absence of pulses. Blood-plasma fraction IV-4 restores, almost completely, the respiratory response to stimulation of cerebral cortex slices treated with these extracts. Further, basic proteins such as histones from calf-thymus nuclei and the protamines, clupein and salmine, inhibit the respiratory response at concentrations as low as 0·1 mg./ml. It was concluded that the inhibitory material of the brain extracts was probably a histone, and that the loss of response in cerebral slices kept in cold media was due to a reaction of the tissue's histones with acidic sites or carriers that are essential to the metabolic response to electrical pulses (McIlwain, 1959).

The aim of the present investigation was to extract basic proteins from nuclei isolated from cerebral cortex slices after two types of treatment *in vitro*: (1) incubation under ordinary conditions, favourable for retention of respiratory response to electrical stimulation; and (2) keeping in cold media and then incubating. It was thought that such studies might demonstrate a loss of nuclear histones in the slices exposed to cold media. If this could be shown, studies on the localization of the site of reaction of basic proteins in extranuclear parts of the neurone should provide useful information on the biochemical events linking electrical stimulation and metabolism. To our knowledge the only study of histones from cerebral tissues, apart from preliminary characterization (Mirsky, 1943), is that of Palladin, Rashba & Shtutman (1951), who reported an arginine content of 12 % and an isoelectric point of pH 10–11 for histones obtained from nuclei of the cerebral cortex of the cow and the dog.

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EXPERIMENTAL

Tissue, media, metabolism

Guinea pigs were used unless otherwise stated; they were stunned by a blow on the neck, the throat was cut to allow bleeding and the brain removed. Cerebral cortex slices, 0·35 mm. thick, were prepared promptly with a razor blade and guide, and after being weighed moist were transferred to phosphate media. Respiration was measured manometrically at 37·5° in the oxygenated phosphate media containing (mM): NaCl, 134; KCl, 5·2; $CaCl_2$, 2·8; KH_2PO_4, 1·3; $MgSO_4$, 1·3; Na_2HPO_4, 10·4; and glucose, 10; pH, 7·4 (Rodnight & McIlwain, 1954). Lactic acid was determined at the end of manometric experiments by Barker & Summerson's (1941) method. Large Warburg flasks (approx. 100 ml. capacity) were used so that a sufficient quantity of tissue slices could be obtained for the extractive procedures. Approx. 500 mg. of cortex slices were incubated with 15 ml. of buffered phosphate media in each flask. Slices to be exposed to cold were placed in the oxygenated media in Warburg flasks in a tray of ice–water which was kept in the refrigerator for 16 hr. The flasks were lightly stoppered and an O_2 atmosphere was not maintained. After exposure to cold, the medium was drained from the slices which were then suspended in fresh media, equilibrated with O_2, and respiratory measurements were made at 37·5°. The O_2 uptake of fresh slices, not exposed to cold media, was determined in parallel.

In studies with unsliced cerebral cortex, as much as possible of the white matter was removed by opening the ventricles and by blunt dissection which removed the layers of subcortical white matter.

Preparation of subcellular fractions

Nuclear fraction. All operations were conducted at 0 to 4°. The medium used for the isolation of nuclei was a slight modification of that of Allfrey, Mirsky & Osawa (1957) and consisted of 0·32 M-sucrose with 3 mM-$CaCl_2$ adjusted to pH 7·0 with NaOH. After brief preliminary dispersion in a glass homogenizer with a loose-fitting polythene plunger, homogenization was carried out in a Model 10 Emanuel–Chaikoff hydraulic tissue homogenizer (Microchemical Specialties Co.) with an orifice of 25 μ. This method gave high yields of intact nuclei, with almost no contamination by cell-bodies, and under conditions that could be precisely reproduced. Medium to tissue ratios (v/w) of 10 gave the best results. The homogenized tissue was filtered through one layer of cotton screen (300 mesh) and two layers of napped flannelette. The filtrate was centrifuged in a Spinco model L ultracentrifuge at 3500 rev./min. (800 *g*)

for 10 min. The sedimented nuclear fraction was washed four times by resuspending in the original volume of homogenizing medium and recentrifuging as before. The washed fraction was finally resuspended twice in 0·14 M-NaCl, pH 7·0, for 10 min. and centrifuged. This procedure extracted nuclear globulins (Allfrey *et al.* 1957). The separation of the nuclei was controlled by examination with a phase-contrast microscope. To achieve better separation of nuclei from cellular particles from slices it was necessary to layer the homogenate on M-sucrose and centrifuge in a SW 25.1 rotor of the Spinco ultracentrifuge for 1 hr. at 24 000 rev./min. (50 000 *g*).

Other fractions. Sucrose–$CaCl_2$ media were unsuitable for preparation of mitochondrial and microsomal fractions because calcium salts increased a tendency of the particles to aggregate and to co-precipitate. These subcellular particles were isolated in media consisting of 0·32 M-sucrose with 0·5 mM-EDTA (ethylenediaminetetra-acetic acid) at pH 7·0 and separated by differential centrifuging based on the method of Brody & Bain (1952). The following fractions were prepared: (1) 'nuclear', after centrifuging at 800 *g* for 10 min.; (2) large 'mitochondrial' after centrifuging at 5000 *g* for 10 min.; (3) small 'mitochondrial' after centrifuging at 20 000 *g* for 10 min.; (4) 'microsomal', after centrifuging at 105 400 *g* for 30 min.

Analyses. The nitrogen content of the various fractions was determined by a micro-Kjeldahl procedure. Deoxyribonucleic acid was determined by the *p*-nitrophenylhydrazine method of Webb & Levy (1955) with a deoxyribose standard.

Extraction and analysis of histones

Two procedures were used for the extraction of histones from isolated nuclei and other fractions of cerebral tissues.

Acid extraction. The procedure of Luck, Rasmussen, Satake & Tsvetikov (1958) for fractionation of calf-thymus histone was used with slight modifications. The washed nuclear and particulate fractions were suspended in 10 vol. of 0·2 N-H_2SO_4 at 0°, agitated for 10 min. with a Vibromix stirrer, kept in the refrigerator for 40 min. and then stirred again for 10 min. The suspensions were then clarified by centrifuging at 31 000 *g* in the cold for 30 min. and the deposit was discarded. The histone sulphates were separated into arginine-rich and presumably lysine-rich fractions by ethanol precipitation in the cold according to the method of Ui (1956). To the clarified solutions absolute ethanol at −10° was added slowly to a final concentration of 20% (v/v), and the arginine-rich histone was allowed to precipitate overnight at −5°. The precipitate was removed by centrifuging at −5° and washed five times with 4 vol. of cold 95% ethanol. Almost all the precipitate dissolved in distilled water, and the little that did not was removed by centrifuging. The histone solution was then freeze-dried to a white powder that was freely soluble in distilled water. The lysine-rich histone was separated by adding absolute ethanol, to a final concentration of 50% (v/v), to the supernatant, after removing the argining-rich histone, and again collected by centrifuging.

In some experiments the acid extract was dialysed against distilled water for 24 hr. before ethanol precipitation. When in trial experiments the histones were extracted as the chloride, the ethanol precipitates were more colloidal and difficult to remove completely by centrifuging. Therefore, all histones were extracted as the sulphate and converted as desired into the chloride by adding an excess of 0·2 M-$BaCl_2$, separating the precipitate by centrifuging, and then dialysing and freeze-drying the supernatant.

Salt extraction. This method was based on those of Crampton, Lipshitz & Chargaff (1954), Butler, Davison, James & Shooter (1954), Smillie, Marks & Butler (1955) and Hnilica (1959) except that the histones were separated from the deoxyribonucleoprotein by 0·2 N-H_2SO_4. To the cold 0·14 M-NaCl-washed nuclear fractions, NaCl was added (10 vol.) to give a final concentration of 1·5 M. The suspension was gently stirred for 4 hr. at 0°. A strong gel of deoxyribonucleoprotein usually formed. The suspension was centrifuged at 50 000 *g* for 30 min. The residue was resuspended in a further 10 vol. of 1·5 M-NaCl and the centrifuging was repeated. To the supernatant, N-H_2SO_4 was added to give a final concentration of 0·2 N. A white precipitate formed. The mixture was kept for 1 hr. in the cold, and the deoxyribonucleic acid sulphate removed by centrifuging. The clear supernatant was dialysed against three changes of distilled water at 4°, filtered to remove insoluble protein, and the histone sulphates were separated into the arginine-rich and lysine-rich fractions by the ethanol precipitation method described above.

Hydrolysis of proteins. Weighed amounts (approx. 2 mg.) of the dried histone sulphates were hydrolysed in 2 ml. of 6 N-HCl for 4 hr. at 110° in sealed tubes with 0·01 ml. of 12·5% (w/v) titanous chloride as catalyst (McIlwain & Hughes, 1944). The hydrolysates were neutralized with NaOH and the precipitate of titanous hydroxide removed by filtration. The precipitate was washed three times with water. The hydrolysate was then made up to standard volumes representing 100–200 μg. of protein/ml.

Determination of arginine. The arginine content of hydrolysates of cerebral histones was determined by the modified Sakaguchi method of Satake & Luck (1958) with *N*-bromosuccinimide instead of hypobromite as the oxidizing agent. A standard series of arginine solutions were run with each hydrolysate. This method gave reproducible colours, provided that the oxidizing agent was rapidly added and the colour read at 500 mμ 5–10 min. after development.

RESULTS

Histones from cerebral cortex

Before extracting histones from cerebral cortex slices incubated under chosen conditions, it was necessary to examine the extractability, yields and some properties of histones prepared from fresh unsliced cerebral cortex. In addition, calf-thymus nuclei were extracted by the same procedure to check the reliability of the methods. Table 1 gives the data obtained. The arginine content of the histone sulphates from cerebral tissues was consistently less than from calf thymus. Salt extraction followed by acid treatment, dialysis and ethanol precipitation in the cold gave slightly higher arginine contents for histone preparations from cerebral tissues, but the yields of histone were lower. Dialysis of acid extracts before ethanol precipitation did not increase the arginine contents nor did reprecipitation with 5 N-ammonia solution. The acid-extraction method, followed by fractional

Table 1. *Arginine content and yields of histone sulphates from cerebral tissues and calf thymus*

For details of separation of nuclear and other fractions, extraction of histones and arginine determination, see Experimental section. Method 1*a* refers to acid extraction followed by precipitation with 20% (v/v) cold ethanol. The method 1*b* refers to precipitation with 50% (v/v) final concentration of the cold ethanol supernatant from 1*a*. Method 2 refers to 1·5 M-NaCl extraction, acid treatment, dialysis and precipitation with 20% (v/v) ethanol. When the number of experiments was over three the arginine value is given ±S.D.; otherwise, each value gives the mean of duplicate determinations on material from one experiment.

Tissue fraction	Method	Yield (mg./g. wet wt. of tissue)	Arginine content (%)
Ox cerebral-cortex nuclei	1*a*	2·6	10·9, 10·5
Ox cerebral-cortex nuclei	1*b*	1·3	1·2, 1·4
Ox cerebral-cortex nuclei	2	0·5	10·5
Ox cerebral-cortex microsomes	1*a*	1·4	5·8, 5·9
Guinea-pig cerebral-cortex nuclei	1*a*	2·7	9·1 ± 0·3 (4)
Guinea-pig cerebral-cortex nuclei	1*b*	1·1	1·6, 3·9, 3·2
Guinea-pig cerebral-cortex nuclei	2	0·7	10·6
Guinea-pig cerebral-cortex microsomes	1*a*	1·6	4·2, 4·1
Human cerebral-cortex nuclei (right superior frontal lobe)	1*a*	2·6	9·2
Calf-thymus nuclei	1*a*	4·2	14·2
Calf-thymus nuclei	1*b*	2·0	4·2

Table 2. *Distribution of nitrogen, deoxyribose and arginine of proteins extracted from subcellular fractions of unsliced guinea-pig cerebral cortex*

Homogenization of 3 g. of cortex was made with an Emanuel–Chaikoff apparatus in 0·32 M-sucrose with 3 mM-$CaCl_2$, pH 7·0. Fractions were separated, acid was extracted and the proteins were precipitated by ethanol, 20% (v/v) at −5° (for details see Experimental section). The mitochondrial fraction was separated by a centrifugal force of 20 000 *g* for 10 min., the microsomal at 105 400 *g* for 30 min. The arginine values marked by an asterisk refer to the protein precipitated by 2·5 vol. of ethanol at −5°, removed by centrifuging, washed and freeze-dried. The arginine values are the means of two determinations.

	N of fraction		Extracted protein		Deoxyribose of fraction	
Tissue fraction	(mg./g. wet wt. of tissue)	(%)	Yield (mg./g. wet wt. of tissue)	Arginine content (%)	(μg./g. wet wt. of tissue)	(%)
Whole homogenate	16·04	100	—	—	1820	100
Washed nuclei	4·47	28	—	—	999	55
Mitochondria	4·13	25	—	—	605	33
Microsomes	1·10	7	—	—	75	4
Supernatant	6·62	41	9·1	3·3*	91	5
Recovery	—	101	—	—	—	97
0·14 M-NaCl washings of nuclei	0·46	3	1·0	3·9*	—	—
First acid extract, nuclei	1·51	9	2·7	9·4	16	1
Second acid extract, nuclei	0·4	2	0·2	7·6	—	—
Acid extract, mitochondria	0·6	3	1·0	8·1	—	—
Acid extract, microsomes	1·13	6	1·5	4·2	—	—

ethanol precipitation in the cold, was the simplest method that gave satisfactory yields of arginine-rich histones, and this method was used for extraction of histones from slices. A minimum of 1·5 g. of guinea-pig cerebral-cortex slices (i.e. three hemispheres) was necessary to give an adequate yield of dry protein (i.e. 4 mg.). Basic proteins, extracted by acid from whole ox brain and from the guinea-pig 'microsome' fractions, consistently contained less arginine than the basic proteins obtainable from the separated nuclei. This finding was important as it enabled comparisons to be made between non-nuclear particulate fractions from slices treated in cold media (see below).

Table 2 shows a representative experiment in which fresh unsliced guinea-pig cerebral cortex was separated into subcellular fractions in sucrose–calcium chloride media, and the nitrogen, the deoxyribose and the arginine of the acid-extractable proteins were determined. When examined under phase contrast, the washed nuclear fraction showed little contamination with mitochondria. The mitochondrial fraction contained considerable numbers of small neuroglial nuclei and this is further reflected in the deoxyribose values. The first acid extraction of the nuclear fraction yielded proteins with the highest arginine content. Acid extraction of the mitochondrial pellet also yielded

basic proteins of high arginine content, but this was due to contamination by small nuclei. The basic proteins from the microsomal pellet were of low arginine content. Protein from the supernatant fraction, precipitated by 2·5 vol. of ethanol in the cold, contained 3–4 % of arginine.

Properties of cerebral cortex histones. Preliminary studies of the properties of the acid-extractable basic proteins were carried out to indicate that histones were in fact being prepared. The objective, at this stage of the study, was to obtain the mixed basic proteins and ensure that the reproducibility of the extraction procedures and the composition of the extracts were sufficient to enable comparisons to be made between extracts from tissue slices with or without pretreatment in cold media.

The undenatured, freeze-dried, cerebral cortex histones were white fluffy powders, soluble in water at neutral and at acid pH, and insoluble in 5 N-ammonia solution. Their nitrogen content was approx. 17 %. On adding dilute alkali to a clear aqueous solution of the protein, clouding commenced at pH 9·0, and reached a maximum at pH 10·0 with no further turbidity above pH 10·5. The isoelectric point thus lies between pH 9 and 10·5. The solid proteins stain intensely with the dye Fast green FCF. The molecular weight of some components of the histones was large enough to prevent the proteins from passing through a cellophan dialysis membrane. Dialysis however did lead to losses of some components and to some formation of insoluble precipitates of material within the sac. The ultraviolet absorptions of guinea-pig and ox cerebral-cortex histones were determined in a Hilger spectrophotometer in 0·01 N-hydrochloric acid. There was no absorption peak at 260 mμ and the ratio of absorption at 260 mμ to that at 280 mμ was 0·7. This indicates no contamination with nucleic acids. For ox cortex histone, $E^{1\%}_{1\,cm.}$ was 5·4 at 280 mμ and 26·6 at 220 mμ; for guinea-pig cortex histone, $E^{1\%}_{1\,cm.}$ was 5·2 and 26·3 at 280 mμ and 220 mμ respectively. Cruft, Mauritzen & Stedman (1958) reported that $E^{1\%}_{1\,cm.}$ was 6·035 at 275 mμ for 98 % pure β-histone from calf thymus.

A qualitative analysis of the amino acids in hydrolysates of the cerebral cortex histones was made by two-dimensional paper chromatography with pyridine–*n*-butan-1-ol–water (1:1:1, by vol.) and butan-1-ol–acetic acid–water (12:3:5, by vol.) as solvent systems. Of the basic amino acids, arginine gave the most intense spot with ninhydrin and Sakaguchi sprays. Other features of the amino acid composition were the absence of cystine and the occurrence of only trace amounts of methionine and taurine. Tyrosine was present. On alkaline hydrolysis there was no tryptophan and the Hopkins–Cole test was negative. Electrophoresis of guinea-pig cerebral-cortex histones in starch gels (0·01 M-acetate buffer, pH 5·0, 120 v, 16 hr., 4°) revealed nine zones in the arginine-rich fraction and two zones in the lysine-rich fraction migrating to the cathode, with one faint zone migrating to the anode. It was apparent that the arginine-rich histones were a mixture of basic components.

Histones from cerebral cortex slices with and without exposure in cold media

The results in Table 2 show that the arginine contents of acid-extractable proteins from nuclei and microsomes, and of the ethanol-precipitable protein from the supernatant fraction, are characteristically different. If a migration of histones from the nucleus to some extranuclear site has occurred during storage in cold media it should be demonstrated by changes in the arginine content of the acid-extractable proteins from the subcellular fractions.

The results of three experiments on sliced cerebral cortex, incubated for 40 min. at 37·5° with and without prior treatment at 0° for 16 hr., are shown in Table 3. Considerable protein was lost from the slices to the phosphate media, both during incubation at 37·5° and on keeping in the cold. Ethanol-precipitated proteins from the incubation media had arginine contents which were similar for slices untreated and treated in cold media. If basic protein is lost from the nuclei of cold-treated slices, it does not appear in significant amounts in the incubation media but remains within the slice. Separation of homogenates of slices into subcellular fractions was considerably more difficult than the corresponding separation of unsliced tissue. The mitochondria were swollen, clumped together and adhering to the nuclei. They were thus co-precipitated with the nuclei on differential centrifuging. Four washings did not give clean nuclear preparations. The contamination of the nuclear fraction by mitochondria and other subcellular particles is shown by the high nitrogen values. The homogenates of slices were accordingly separated into three fractions: (1) a 'nuclear' fraction after centrifuging at 800 ***g*** for 10 min., (2) a 'particulate' fraction after centrifuging at 105 400 ***g*** for 30 min. and (3) a supernatant. The particulate fraction contained small mitochondria and microsomes but the yield of acid-extractable protein from this fraction was too small for measurements of arginine content to be made.

Table 3 shows that the basic proteins from the 'nuclear' fractions of cold-treated slices are both less in quantity and contain considerably less arginine than the same fractions from untreated slices. However, the arginine content of proteins precipitated from the supernatant is higher in cold-treated slices. These findings suggest that arginine-rich protein has been lost from the nuclei of these

slices and has in part appeared in the supernatant fraction. They do not rule out the possibility that the basic protein may have left the nuclei and become fixed to other subcellular particles. The nuclear fraction, derived from tissue kept in cold media, is contaminated with other subcellular particles and consequently if basic protein was attached to these particles they would be acid-extracted along with the nuclei. For this reason it was decided to attempt cleaner separations of the nuclei from sliced tissues by layering the 0·32 M-sucrose homogenate over M-sucrose and centrifuging at 50 000 *g* for 1 hr. After centrifuging, the 0·32 M-sucrose layer and also the M-sucrose layer were pipetted off, leaving a small pellet in the bottom of the centrifuge tube. Samples from the upper and lower layers and from the pellet were examined microscopically under phase contrast. The upper layer contained most of the nuclei with fragments of myelin and other membranes. The nuclei from slices that had been pretreated at 0° contained no nucleoli and were not granular in appearance, although the nuclei were intact and normal in shape. Nuclei from untreated slices contained nucleoli, but were swollen and showed a fine granularity to the nucleoplasm. The lower layer contained mitochondria, many small particles and

Table 3. *Guinea-pig cerebral-cortex slices untreated and pretreated in cold media: respiration, arginine content of proteins from incubation media and subcellular fractions, and nitrogen distribution*

Slices were prepared and respiration was determined with and without pretreatment at 0° for 16 hr. in buffered phosphate–glucose media. After respiratory measurements, slices were drained, homogenized and separated into the 'nuclear' fraction (800 *g*, 10 min.) and 'particulate' fraction (105 400 *g*, 30 min.), and the fractions were acid-extracted. The proteins were precipitated by ethanol (20%, v/v) at −5°, and their arginine was determined by a modified Sakaguchi reaction. For details see the Experimental section. Proteins in the incubation media, supernatant and NaCl washings were precipitated by 2·5 vol. of ethanol at −5°. Arginine values are the mean of duplicate determinations in three separate experiments. Respiration and lactate formation are expressed as mean values ±S.D. with the number of measurements in brackets.

	A. From fresh slices incubated at 37·5° for 40 min.		*B*. From slices kept at 0° before incubation as in *A*	
Material	N of material (mg./g. fresh wt. of tissue)	Arginine of extracted protein (%)	N of material (mg./g. fresh wt. of tissue)	Arginine of extracted protein (%)
Whole homogenate	14·6	—	13·2	—
Medium of 0° exposure	—	—	2·8	3·4, 2·8, 3·5
Incubation media	1·4	2·5, 2·3, 2·6	1·7	2·6, 2·1, 2·0
Nuclear fraction	7·9	—	7·4	—
Particulate fraction	1·7	—	1·0	—
Supernatant	3·3	4·4, 3·3, 3·8	2·4	6·6, 6·1, 5·1
0·14 M-NaCl washings	0·5	5·5, 6·0, 6·1	0·6	6·2, 4·7, 5·0
Acid extract of nuclear fraction	2·1	8·6, 9·0, 8·3	0·9	5·8, 6·4, 5·5

	A. From fresh slices incubated at 37·5° for 40 min.		*B*. From slices kept at 0° before incubation as in *A*	
	Respiratory rate (μmoles of O_2/g./hr.)	Lactic acid accumulating (μmoles/g./hr.)	Respiratory rate (μmoles of O_2/g./hr.)	Lactic acid accumulating (μmoles/g./hr.)
Slices before homogenizing	57 ± 3 (6)	28 ± 5 (4)	42 ± 4 (6)	31 ± 6 (4)

Table 4. *Arginine contents of acid-extracted proteins from isolated nuclei and other particles of cerebral cortex slices untreated and pretreated by cold media*

Slices of guinea-pig cerebral cortex (approx. 1·5 g.) after incubation at 37·5° for 40 min. were homogenized and layered on M-sucrose in the SW 25.1 rotor of a Spinco ultracentrifuge and spun at 50 000 *g* for 1 hr. The upper and lower layers were pipetted off. Under phase contrast, the upper layer contained mainly nuclei and the lower layer mainly small particles with a few nuclei. The lower layer was diluted to 0·3 M-sucrose and both layers were spun at 40 000 rev./min. for 30 min. in the 40 rotor. The pellets were extracted with 0·2 N-H_2SO_4, the proteins were separated, and the arginine content was determined as outlined in the Experimental section. Arginine values are the mean of two determinations.

	Extracted material (mg.)		Arginine content (%)	
Layer extracted	Slices not exposed at 0°	Slices exposed at 0°	Slices not exposed at 0°	Slices exposed at 0°
Upper layer (mainly nuclei)	2·0	No protein obtained	8·7	—
Lower layer (mainly other particles)	1·6	1·5, 1·8	5·7	9·7, 9·4

a few small nuclei which had thus passed through the M-sucrose layer. The upper layer and the lower layer diluted to 0·3M-sucrose were centrifuged at 105 400 *g* for 1 hr., and the pellets acid-extracted. The results are shown in Table 4. No basic protein could be extracted from the layered nuclei from slices pretreated in cold media. Untreated slices contained nuclear basic proteins of arginine content similar to unsliced tissue (see Table 2). The particles, in the lower M-sucrose layer, from cold-treated slices contained basic protein of high arginine content. The basic proteins extracted from the lower layer particles from slices not subjected to cold media contained less arginine, approaching rather the values obtained for microsomes from fresh cortex (see Table 1).

These experiments indicate a complete loss of basic protein from the nuclei of cold-treated slices and the appearance of an arginine-rich, acid-extractable protein in the small-particle fraction. Further qualitative evidence for a loss of nuclear constituents in cold-treated slices was obtained by observing the effects of 2M-sodium chloride on isolated nuclei. When nuclei from fresh slices were suspended in 2M-sodium chloride and examined under phase contrast, a halo of extracted deoxyribonucleoprotein was visible (see Philpot & Stanier, 1956). Nuclei obtained from slices kept in the buffered phosphate–glucose media at 0° for 16 hr., and then suspended in 2M-NaCl, showed no nucleoprotein halos and did not form gels.

Combination of microsomal particles with histones

With the object of localizing more specifically the type of subcellular particles that might be involved in the binding of histones released from nuclei under adverse conditions, properties of freshly prepared subcellular fractions from unsliced cerebral cortex tissue were studied. In particular, their ability to remove histones from solution was examined. In these experiments weighed samples from separated subcellular pellets were suspended in phosphate buffer (0·001M, pH 7·0) containing a known amount of histone and the suspension was clarified by centrifuging at 105 400 *g* for 30 min. Minimal salt concentrations were employed in these experiments to avoid precipitation or flocculation of the histone or the particles by the buffer alone. After centrifuging, measured portions of the supernatant solutions were hydrolysed and their arginine content was determined. The results are shown in Table 5. It was observed that when the microsomal fractions were suspended with histones from nuclei of the cerebral cortex itself or from calf thymus, flocculation occurred. The results in Table 5 show clearly that the microsomal fraction is the most active in combining with histones and removing them from solution.

Experiments were performed in which the microsomal fraction was suspended in protein solutions containing crystalline bovine albumin, γ-globulin (Cohn fraction II, Armour Laboratories) and purified gelatin at concentrations of 1 mg./ml. in phosphate buffer (0·001M, pH 7·0). After the suspension had been clarified by centrifuging at 105 400 *g* for 30 min. the protein content in a sample of the supernatant was determined. Under these conditions bovine plasma albumin and gelatin were not adsorbed by the particles and only 5% of the γ-globulin was removed from solution. These

Table 5. *Combination of subcellular particles from cerebral cortex with histones*

Subcellular fractions were separated from fresh cortex and homogenized in 0·32M-sucrose with 0·5M-EDTA, at pH 7·0, as outlined in the Experimental section. Samples (200 mg.) of the particulate pellets were suspended with histone solutions in 2 ml. plastic tubes for 10 min. Histone solutions contained 1 mg. of dried protein/ml. in mM-phosphate buffer at pH 7·0. The suspensions were centrifuged at 105 400 *g* for 30 min. in the 40 rotor of the Spinco ultracentrifuge. Samples of supernatants were taken for hydrolysis of the protein remaining, and arginine was determined in the hydrolysate. Arginine values lower than the control solution containing no added particles indicate removal of histone from solution.

Tissue fraction	Centrifugal force used in preparation		Arginine content of supernatant after suspension with 200 mg. of fraction (μg./ml.)	
	g	min.	Suspended with thymus histone	Suspended with guinea-pig cerebral-cortex histone
Nuclei	800	10	127	83
Large mitochondria	5 000	10	132	90
Small mitochondria	20 000	10	83	60
Microsomes	105 400	30	22	27
Histone alone	105 400	30	143	101

experiments indicate that the removal of proteins from solution by microsomal particles is to some extent specific for basic proteins and is not due to non-specific protein adsorption.

DISCUSSION

The suggestion (McIlwain, 1959), that histones leave their normal nuclear site when cerebral cortex slices are kept in buffered glucose media in the cold, has been shown in the present study to be correct. Nuclei from cold-treated slices yielded no arginine-rich basic proteins, but a particulate fraction from these slices containing predominantly small mitochondria and microsomes is considerably richer than one from untreated slices in these proteins. Further, freshly prepared microsomal fractions contain the particles most active in combining at neutral pH with histones and removing them from solution. It is concluded that in cold-treated slices the histones from the nuclei have combined with some component of the microsomal fraction. It appears likely that this combination is the basis of the loss of responsiveness to electrical stimulation, both in tissues kept in cold media and in normal tissues to which histones and protamines have been added as such.

The nuclear membrane is known to be permeable to some proteins (see Roodyn, 1959). Protamine and histone solutions have been observed to pass easily into isolated rat-liver nuclei (Anderson & Wilbur, 1952; Philpot & Stanier, 1957). Pollister & Leuchtenberger (1949) reported a 75 % loss of protein from guinea-pig nuclei immersed in physiological saline at 5° for 3 hr. without any changes in the microscopic appearance of the nuclear membrane. Recent studies of nuclear structure with the electron microscope have shown that the nuclear membrane has a fenestrated appearance and contains 'pores' and 'annulae' ranging from 250 to 1000 Å in diameter (Watson, 1954, 1959; Beams, Tahmisian, Devine & Anderson, 1957). If these structures are in fact true pores they are large enough to allow passage of proteins, particularly histones, if their associations within the nucleus are disrupted.

Histones are regarded as existing in the nucleus as salt-like compounds with deoxyribonucleic acid (for recent observations, see Crampton, 1957; Zubay & Doty, 1959). The ability to form gels, by cross-linkages between the long nucleohistone units, is a good indication of the integrity of the deoxyribonucleoprotein of the nucleus, and it has been shown that deoxyribonucleases prevent this gel formation (Dounce, 1955; Dounce, O'Connell & Monty, 1957). Isolated liver nuclei are permeable to these enzymes which affect their microscopic appearance (Anderson, 1953). Philpot & Stanier (1956) showed that the optical and size changes, seen under phase contrast in nuclei placed in various media, were due to alterations in the nucleohistone gel and not to tonicity effects. The inability of nuclei from cerebral cortex slices kept in cold media to form nucleohistone gels, and the absence of granularity of the nucleoplasm, is further evidence for a dissociation of nucleic acids and histones. Whether this is due to a direct effect of cold, to the action of nucleases or is a result of the swelling of cerebral tissues in buffered, iso-osmotic glucose media (Pappius & Elliott, 1956) is unknown; more than one of these factors may be involved. Any specific localization requiring metabolic maintenance may be suspected to become defective in cold tissue, thus allowing the distribution of cell constituents to be conditioned to a greater extent by purely chemical affinities. The affinity for basic proteins of sites outside the nucleus is clearly shown by the results of Table 5. However, for development of the unresponsive state at 0° it was necessary that the tissue should be in contact with fluid media. The tissue under these conditions imbibes fluid, which may thus provide additional channels for movement of tissue constituents.

Of particular interest is the demonstration that microsomal fractions are the most active in binding histones. It is possible that the anionic sites, previously postulated (McIlwain, 1959) as being involved in the response of tissues to electrical excitation, are located in this fraction and are the entities inhibited by combination with histones and protamines. Basic proteins may be useful substances to aid in the characterization of the acidic, microsomal components. Basic proteins, particularly protamines, are known to form complexes with acidic substances of large molecular weight (e.g. insulin, heparin, phosphorylase and acidic mucopolysaccharides) and to interact with serum proteins (Portman & Holden, 1959; Rubini, Becker & Stahmann, 1953; Madsen & Cori, 1954; Bollet, Seraydarian & Simpson, 1957; Carroll, Callanan & Saroff, 1959; Truffet, de Gennes, Robert & Polonovski, 1958). The microsomal fraction of tissue homogenates, particularly of cerebral tissues, is heterogeneous but has been shown under the electron microscope to consist essentially of fragmented ergastoplasmic elements (Chauveau, Gautier, Moulé & Rouiller, 1955). In nerve cells the ergastoplasm is most conspicuous and is orientated in areas corresponding to the Nissl bodies. It consists of a continuous system of intracytoplasmic membranes, connected both to the nucleus and to the outer cell membrane, and small dense ribonucleoprotein particles closely associated with the membranes (Palade & Porter, 1954; Palay & Palade, 1955; Haguenau, 1958; Watson, 1955; De Robertis, 1954). Evidence for the possibility that the acidic

sites responsible for binding basic proteins are located within these membrane structures is being sought.

SUMMARY

1. Histones were extracted from isolated nuclei of unsliced and sliced cerebral cortex and some of their properties studied. The arginine-rich histone contains at least nine components. The arginine content is 10–11 %. Basic proteins from microsomal fractions contain 4–6 % of arginine.

2. Nuclear histones are lost from nuclear fractions made from sliced cerebral cortex, which has been kept in cold buffered glucose media. Arginine-rich proteins can then be extracted from other subcellular particles of the tissue. Some basic protein also appears in the soluble supernatant proteins. No loss of histones occurred in nuclei from slices not subjected to cold media.

3. Isolated cerebral microsomal fractions bind histones extracted from cerebral cortex nuclei; they also bind calf-thymus histone.

4. It is suggested that the migration of the histone from the nuclei of slices kept in cold media and its combination with acidic microsomal constituents is involved in the concomitant loss of the tissues' response to electrical excitation.

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The Metabolism of Griseofulvin in Mammals

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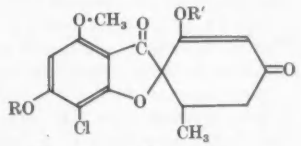
Griseofulvin [7-chloro-4:6:2′-trimethoxy-6′-methylgris-2′-en-3:4′-dione (I:R, R′, Me)], a product of *Penicillium griseofulvum* Dierckx, was first isolated by Oxford, Raistrick & Simonart (1939): its structure was first established by Grove, Ismay, MacMillan, Mulholland & Rogers (1951). This compound has fungistatic activity and was reported to control experimental ringworm in guinea pigs (Gentles, 1958) and in calves (Lauder & O'Sullivan, 1958) when administered orally.



(I)

Reports of its successful use in the treatment of human ringworm infections came from Williams, Marten & Sarkany (1958), Blank & Roth (1959) and Riehl (1959). During work on the distribution and excretion of [^{36}Cl]griseofulvin after oral administration, we found that a large amount of radioactive material was excreted in the urine of test animals, but that most of this was biologically inactive and only a very small percentage of it griseofulvin. The major metabolite present in the urine has been characterized as 7-chloro-6-hydroxy-4:2′-dimethoxy-6′-methylgris-2′-en-3:4′-dione (I:R, H; R′, Me) (6-demethylgriseofulvin).

EXPERIMENTAL

Material. [^{36}Cl]Griseofulvin was prepared by fermentation in a medium containing K^{36}Cl. Its specific activity was 2·36 μc/m-mole, and it gave 4240 counts/min./mg. on a thin-window Geiger–Müller counter. Samples were plated on 1 cm.^{2} planchets; as great accuracy was not essential, samples of extracts and column eluates were directly applied to the planchets, which were then dried in an oven before counting. All samples were prepared by plating less than 15 mg. of material; below this weight self-absorption errors were negligible. All results, unless otherwise stated, were obtained by this method, but have been expressed as percentages of the original activity.

Paper chromatography. The mobile solvent system used was the organic phase of benzene–*cyclo*hexane–methanol–water (5:5:6:4, by vol.) to which 0·5% of acetic acid had been added after separation. When run on Whatman no. 1 paper by descending chromatography, griseofulvin appeared as a bright fluorescent spot under ultraviolet light R_F 0·9, and the material subsequently shown to be 6-demethylgriseofulvin appeared as a darker-blue fluorescent spot, R_F 0·15. Chromatograms were run for 6 hr. at 24°.

Spectrometry. All ultraviolet spectra were determined in 80% ethanol to which was added 1% (v/v) of either 2N-HCl or 2N-NaOH. Infrared spectra were determined in $CHBr_3$, with a Perkin–Elmer model 21 instrument with a NaCl prism.

Extraction of urine

Rabbit. Two rabbits (total wt. 4 kg.) were each given a single oral dose of 500 mg. of [^{36}Cl]griseofulvin/kg., prepared by diluting our [^{36}Cl]griseofulvin 1 in 20 with non-radioactive griseofulvin, and their urine was collected. The urine excreted in the first 24 hr. after dosing (bulked volume 150 ml.) contained 11·2% of the radioactivity administered and the 24–48 hr. urine (310 ml.) contained 24%. The 24–48 hr. urine at pH 6·5 was exhaustively extracted with 300 ml. volumes of butan-1-ol, which removed 85% of the radioactive material. Subsequent paper chromatography of the aqueous residue showed no trace of 6-demethylgriseofulvin. A dark sticky solid (4·8 g.) was obtained on evaporation of the combined butan-1-ol extracts; this was extracted with $CHCl_3$, first by heating under a reflux condenser and then in a Soxhlet extractor. By these methods 92% of the radioactivity was taken into the $CHCl_3$, leaving behind about 3·8 g. of solid. The combined $CHCl_3$ extracts were concentrated to 5 ml. and run through a column of 30 g. of silica gel. (British Drug Houses Ltd.) prepared from a $CHCl_3$ slurry. The fractions from this column, which removed almost all the coloured impurities, were each checked for amount of radioactivity, and fractions 1 and 2, eluted from the column with 75 ml. and 50 ml. of $CHCl_3$ respectively, contained 70% of the input. The solid in these fractions was dissolved in a small volume of ethanol and precipitated as crystals by addition of benzene to the hot solution. These crystals (235 mg.) had approximately the same specific activity as the original [^{36}Cl]griseofulvin. Paper chromatography on further fractions from this column, eluted with ethanol–$CHCl_3$ (1:1, v/v) and finally with ethanol, showed that they contained small quantities of the same material, although they could not be crystallized. The crystalline material from the column was recrystallized from ethanol–2N-acetic acid (1:8, v/v) and then from ethyl acetate–light petroleum (b.p. 60–80°) until a constant m.p., 273–5° (decomp.) uncorr., was reached (Found: C, 57·0; H, 4·5; OMe, 18·4. $C_{16}H_{15}O_6Cl$ requires C, 56·7; H, 4·4; OMe, 18·3%.) Light-absorption max. in acid soln. 235·5 and 293·5 mμ

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(log ϵ 4·41 and 4·44 respectively); in alkaline soln. 249·5 and 330 mμ (log ϵ 4·42 and 4·58 respectively).

Rat. Rats, given oral doses of 100 mg. of [^{36}Cl]griseofulvin/kg., excreted in the urine 10·4% of the radioactivity administered in the first 24 hr. and 4·2% in the next 24 hr. The 0–24 hr. urine (300 ml. from 24 rats) adjusted to pH 5 with 0·1 N-HCl, was extracted with 3 × 0·33 vol. of $CHCl_3$. The combined extracts contained 65% of the original radioactivity of the urine, only 4% being present in the final extract. As in the extraction of rabbit urine, the $CHCl_3$ extract was purified on a silica-gel column, and a crystalline solid was obtained from the first two fractions. These crystals (15 mg.) had the same ultraviolet spectra and R_F as the crystalline material from rabbit urine and gave no depression of melting point when mixed with it. A portion of the $CHCl_3$ extracts was examined by paper chromatography. The spot corresponding to 6-demethylgriseofulvin, when cut out from the paper and counted, was equivalent to only about 45% of the original urine activity.

Human urine. One day's urine (pH 4·5, 1260 ml.) from a patient, taking a daily oral dose of 1 g. of griseofulvin, was extracted with 3 × 600 ml. of $CHCl_3$. These extracts were purified on a silica-gel column and crystallized as before; 105 mg. of crude crystals having the same properties as 6-demethylgriseofulvin were obtained.

To check that the 6-methylgriseofulvin did in fact appear free in the urine, samples of the fresh urine from the three species were chromatographed and gave a 6-demethylgriseofulvin spot which was radioactive. In all urine samples small amounts of griseofulvin were detected, but always less than 1% of the dose administered.

Demethylation of griseofulvin by rat-liver slices

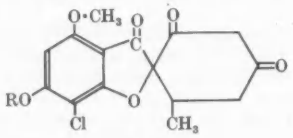
Rat-liver slices (3 g.) were incubated at 37° in 150 ml. of Krebs bicarbonate solution (6·9 g. of NaCl, 0·35 g. of KCl, 0·28 g. of anhydrous $CaCl_2$, 2·1 g. of $NaHCO_3$ and 0·16 g. of anhydrous KH_2PO_4 made up to 1 l. with water) containing 30 μg. of griseofulvin/ml.; $O_2 + CO_2$ (19:1) was bubbled through the solution continuously. After 4 hr. the mixture was filtered through cotton wool and the filtrate was acidified to pH 1·5 with 2 N-HCl. It was then extracted with 3 × 1 vol. of ethyl acetate, and the extract dried over anhydrous Na_2SO_4 and concentrated to 4 ml. When a portion of this was run on a paper chromatogram, spots corresponding to both griseofulvin and 6-demethylgriseofulvin were observed. The remainder of the material in the ethyl acetate was transferred to 80% ethanol and showed the same change in ultraviolet spectrum in acid and alkaline solution as 6-demethylgriseofulvin.

Preparation of griseofulvin from 6-demethylgriseofulvin

6-Demethylgriseofulvin (5 mg.) was treated with a solution of diazomethane in ether. After 2 hr. the ether and excess of diazomethane were evaporated off and the residual solid was recrystallized from ethanol. The crystals had the same R_F as griseofulvin and showed no depression of melting point when mixed with an authentic sample.

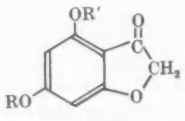
DISCUSSION

Structure of 6-demethylgriseofulvin (I: R, H; R′, Me). Microanalysis showed that this compound differed only by one carbon and two hydrogen atoms from griseofulvin; that a methyl group had been replaced by hydrogen at one of the methoxyl groups was confirmed by conversion of the material into griseofulvin by reaction with diazomethane. Of the three possible monodemethylgriseofulvins only griseofulvic acid (II: R, Me) was known (Grove, MacMillan, Mulholland & Rogers, 1952), and

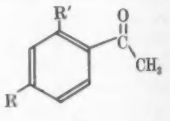


(II)

this material differed in m.p. and ultraviolet spectrum from the compound extracted from urine. Characterization of the excretion product has been obtained from spectroscopic evidence. Duncanson, Grove, MacMillan & Mulholland (1957) found that the methyl ether linkage in position 6 of griseofulvin was more labile to aqueous alkali than that in position 4. These workers found in the spectra of a series of hydroxycoumaranones (III) and Cram & Crantz (1950) found in a series of hydroxyacetophenones (IV) that in compounds with a hydroxyl



(III)



(IV)

group *para* to the carbonyl (III: R, H; R′, Me; IV: R, OH; R′, H) the K band of the anion occurs at a longer wavelength and is more intense than that of the undissociated molecule, whereas when the hydroxyl group is *ortho* to the carbonyl (III: R, Me; R′, H; IV: R, H; R′, OH) very little change in the K band takes place (Table 1). Duncanson *et al.* (1957) found that the infrared spectrum of 4-hydroxy-6-methoxycoumaranone (III: R, Me; R′, H) has a carbonyl frequency 18 cm.$^{-1}$ lower than that of 4:6-dimethoxycoumaranone (III: R, R′, Me), that of 6-hydroxy-4-methoxycoumaranone (III: R, H; R′, Me) being only 2 cm.$^{-1}$ lower. The carbonyl frequency for the metabolite of griseofulvin is only 1 cm.$^{-1}$ lower than that for griseofulvin. The spectra of the excretion product therefore are in accord with the structure 6-demethylgriseofulvin (I: R, H; R′, Me).

Metabolism of griseofulvin. From the results summarized in Table 2 it is clear that 6-demethylgriseofulvin is the major identified metabolite. It is possible that some of the material remaining in

Table 1. *Comparison of the ultraviolet spectra of model compounds and the urine metabolite*

Compound	Acid solution		Alkaline solution	
	$\lambda_{max.}$ (mμ)	log ε	$\lambda_{max.}$ (mμ)	log ε
(III): R, Me; R′, H	280	4·31	285	4·35
(III): R, H; R′, Me	284	4·35	318	4·53
(IV): R, H; R′, OH	252	3·97	257	3·76
(IV): R, OH; R′, H	277	4·16	325	4·36
(II): R, H	292	4·30	327	4·65
(I): R, Me; R′, Me	293	4·39	293	4·39
Urine metabolite	293·5	4·44	330	4·58

Table 2. *Summary of metabolism of griseofulvin*

Species	Dose (mg./kg. body wt.)	Period of urine collection (hr.)	Per cent of dose excreted in urine		
			Total	As 6-demethyl-griseofulvin	Unchanged
Rabbit	500	0–48	35·2	27·5	< 1
Rat	100	0–48	14·6	6·5	< 1
Man	17*	24	—	10·5	—

* This dose had been taken daily for 30 days.

the urine after exhaustive extraction with butanol or chloroform may be conjugated either as the glucuronide or sulphate of this metabolite.

The metabolic fate of some substituted aromatic alkyl ethers is known to involve a preliminary dealkylation. For example, substituted anisoles (Bray, Craddock & Thorpe, 1955) are converted into substituted phenols in the body. This work has been extended by Axelrod (1956), who has shown that the dealkylation taking place in the liver is caused by an enzyme system found in the microsomes of liver cells, and that for full activity this microsomal system requires the presence of reduced triphosphopyridine nucleotide and oxygen. That this cleavage takes place by an oxidative mechanism was demonstrated by the formation of formaldehyde. Of all the substituted anisoles studied in these experiments, the rate of dealkylation of *p*-methoxybenzaldehyde was among the greatest and of the three methoxybenzoic acids tested *p*-methoxybenzoic acid was metabolized about 20 times as fast as the *o*-isomer. These results help to confirm the structure proposed for the metabolite of griseofulvin. Previously, only quantitative work has been reported on the metabolism of griseofulvin (Bedford *et al.* 1960). These workers demonstrated rapid disappearance of griseofulvin from the blood which they suggested was due to distribution into the tissues followed by metabolic inactivation in the liver.

SUMMARY

1. The urine of animals given oral doses of griseofulvin contains little unchanged griseofulvin. A major metabolite has been characterized as 7-chloro-6-hydroxy-4:2′-dimethoxy-6′-methylgris-2′-en-3:4′-dione (6-demethylgriseofulvin).

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Cardiolipin and Other Phospholipids in Ox Liver

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The objects of this work were to find whether the polyglycerophosphatide detected in liver extracts had the same structure as cardiolipin isolated from ox-heart muscle, and to examine the fatty acid composition of the phospholipids. The molar ratios glycerol:phosphorus:fatty acid reported for the glycerophosphatide (McKibbin & Taylor, 1952; Dawson, 1958; Marinetti, Erbland, Albrecht & Stotz, 1957) differed from those found for cardiolipin by Pangborn (1947), who considered cardiolipin to be a fully acylated polyglycerophosphatide with four glycerol, three phosphoric acid and six fatty acid residues. However, evidence in favour of the structure bis(diacylglycerophosphoryl)-glycerol (diphosphatidylglycerol) for cardiolipin was obtained by oxidative degradation of the deacylated polyglycerophosphate, and by the identification of diglycerides and glycerol diphosphate as the main products of hydrolysis of cardiolipin in 90% acetic acid (Macfarlane, 1958; Macfarlane & Wheeldon, 1959).

The amount of polyglycerophosphatide isolated by McKibbin & Taylor (1952) was about 0·5% of the total phospholipid. Hanahan, Dittmer & Warashina (1957) fractionated the phospholipids of rat and ox liver on silicic acid and found about 2% of the total phosphorus in a fast-running nitrogen-low fraction and about 7% as inositide. While the present work was in progress, Faure, Morelec-Coulon, Maréchal & LeBorgne (1959) isolated from ox liver, by solvent fractionation, a polyglycerophosphatide with an iodine value and a serological activity similar to that of cardiolipin, and with molar ratios of glycerol:phosphorus:fatty acid of 3:2:4; they made no comment on the structure and considered that the acidic phospholipid fraction contained phosphatides other than cardiolipin and phosphatidylinositol. The amount of cardiolipin and inositide obtained by these authors was apparently equivalent to about 1·1 and 1·4% respectively of the total phospholipid phosphorus, on average figures for water and lipid-phosphate content of liver. At the same time Hübscher & Clark (1959) reported briefly the isolation from ox liver of phosphatidic acid, in a yield of 0·5–1·0% of the total lipid phosphorus; this is of considerable interest since, although other workers have not found phosphatidic acid in tissue extracts, Hokin & Hokin (1958) detected its presence by heavy labelling with ^{32}P *in vivo*.

In an attempt to resolve some of the discrepancies, the acidic phospholipids were fractionated by chromatography on silicic acid and also by solvent fractionation of the free acids on the lines used by Faure *et al.* (1959).

By both methods, described below, cardiolipin was isolated in amounts equivalent to about 1–2% of the total phospholipid phosphorus, but unequivocal evidence for the presence of naturally occurring phosphatidic acid was not obtained. The discrepancy between these results and those of Hübscher & Clark (1959, 1960) is discussed later.

METHODS

Analytical methods for P, N, amino N, inositol, glycerol, fatty acid esters (colorimetric), fatty aldehyde, iodine values, cholesterol, phosphomonoester by enzymic assay and ester phosphatide were those used by Gray & Macfarlane (1958). The ratios of constituents are given on a molar basis.

Chromatography of phospholipids on silicic acid-impregnated paper was done according to Marinetti, Erbland & Kochen (1957) at 2° with di*iso*butyl ketone–acetic acid–water (40:20:3, by vol.). In this system the cardiolipin spot appears in front of and separated from the phosphatidylethanolamine spot, corresponding in position with spots 6 and 5, Fig. 1A of Marinetti, Erbland & Kochen (1957); the phosphatidylserine and phosphatidylinositol spots corresponded approximately with spots 4 and 1 in this Figure. Cardiolipin prepared from heart muscle and analysed samples of other phospholipids were used as markers. Paper chromatography of water-soluble phosphoric esters, obtained by mild alkaline hydrolysis, was carried out by Dr L. W. Wheeldon according to Dawson (1954). The relative R_F values in phenol saturated with 1% NH_3 in water were: glycerophosphorylinositol, 0·09; polyglycerophosphate from cardiolipin, 0·13; glycerophosphorylserine, 0·20; glycerophosphate, 0·25; glycerophosphorylethanolamine, 0·67; in *tert.*-butanol–water (62:38, v/v), trichloroacetic acid (10%, w/v); glycerophosphorylinositol, 0·26; glycerophosphorylserine and glycerophosphorylethanolanine, 0·41; polyglycerophosphate from cardiolipin, 0·45; glycerophosphate, 0·61.

Chromatography of lipid extracts on silicic acid columns was done as in Gray & Macfarlane (1958). Gas chromatography of the methyl esters of fatty acid mixtures was carried out by Dr G. M. Gray; the saponification of the phospholipid and preparation of the methyl esters were done as described by Gray (1960).

Identification of the fast-running glycerophosphatide in silicic acid fractionation of ox-liver extract L_1 *as cardiolipin*

Minced ox liver (5·5 kg.) was extracted first with 2 l./kg. and then with 0·5 l./kg. of chloroform–methanol (1:1, v/v) by stirring for 10–15 min. at room temperature with cold solvent. The chloroform layer was washed three times with 0·5 vol. of water, cooled to −10°, filtered from ice and fluff and dried with $CaSO_4$. The yield of lipid in the washed extract (L_1) was equivalent to 28·2 g. dry wt. and 890 mg. of P/kg. of fresh liver; P, 3·1% dry wt.

Portions of L_1 containing 320 mg. of P were chromatographed on columns of 200 g. of Mallinckrodt silicic acid (100 mesh, diam. 5·5 cm., height 15 cm.). The columns were developed first with about 2 l. of chloroform, to remove the neutral lipid, and then with chloroform–methanol (95:5, v/v), to elute the low-N fraction (fraction A). The runs did not separate the N-free phospholipid cleanly; it was sandwiched between a slow tail of the non-P lipid and a fast-running front of the kephalin band, and some was undoubtedly lost in the kephalin front. The kephalin, main lecithin and mixed lecithin and sphingomyelin fractions were eluted by changing the solvent in stages up to chloroform–methanol (20:80, v/v); these fractions contained about 32, 1, 53 and 10% of the total lipid respectively. [Phosphatidylinositol had been removed by the washing of the chloroform extract.]

Identification of cardiolipin in fraction A. Four lots of fraction A containing 18 mg. of P (a yield of 1·8% of the total lipid P) were pooled. The material was very dark-coloured; P, 2·5% on dry wt.; ratio Na:amino N:fatty acid ester:P, 0·11:0·12:1·7:1·0. After hydrolysis in 90% acetic acid at 50° and addition of Feulgen reagent in the usual colorimetric estimation of fatty aldehyde, a purple colour developed which was not extracted into octan-2-ol but remained in particles at the interface. This atypical reaction is discussed below. A sample of fraction A was saponified in methanolic 0·2N-NaOH for 15 min. at 38°. Paper chromatography of the water-soluble ester showed a large spot corresponding to that of the polyglycerophosphate from authentic cardiolipin, small glycerophosphorylserine and glycerophosphorylethanolamine spots and a faint glycerophosphate spot; treatment of the water-soluble ester with bone phosphomonoesterase liberated less than 5% of the total P as orthophosphate; the content of phosphatidic acid, which gives rise to glycerophosphate on deacylation, was therefore low.

Fraction A (15 mg. of P) in 5·0 ml. of ether was brought to about pH 6 with ethanolic 0·2N-NaOH; the solvent was removed *in vacuo* at low temperature and the residue redissolved in 3·0 ml. of ether and twice precipitated with acetone at −10°. The precipitate (P, 10 mg., ratio, amino N:P, 0·05:1·0) was dissolved in 5·0 ml. of ether and 50 ml. of ethanol was added with warming at 38°; after 48 hr. at 2° the colourless solution AS (4 mg. of P) was recovered by centrifuging.

This ethanol-soluble sodium salt contained 3·7% of P on dry wt.; ratio glycerol:P:fatty acid ester, 1·4:1·0:2·1. A portion (62 mg.) of AS was saponified in ethanolic 0·2N-KOH for 2 hr. at room temp. and at 2° overnight. The fatty acids recovered weighed 45 mg. (74% on dry wt.); iodine value 132. The ethanol-insoluble potassium salt of the phosphoric ester was collected by centrifuging, washed once with ethanol, dried and dissolved in water; hydrolysis of a sample with phosphomonoesterase liberated less than 5% of the P as orthophosphate; oxidation with $NaIO_4$ at 20° for 30 min. gave 0·97 mole of formaldehyde/mole of P. This agrees with the structure of three glycerol residues linked by two phosphoric acid groups (Macfarlane, 1958). On treatment with periodate at 100° in N-H_2SO_4 for 1 hr. the apparent content of glycerophosphate (Burmaster, 1946) was 42% of the total P; with prior hydrolysis of the ester in N-HCl at 100° for 10 min. the value was 91%; this behaviour corresponds with that of the polyglycerophosphate from cardiolipin.

It was concluded that a polyglycerophosphatide, with a structure similar to that of cardiolipin, was the main phospholipid component in the fast-running fraction. This is in contrast to the findings of Hübscher & Clark (1960). The recovery of the soluble sodium salt, however, was low, and the ethanol-insoluble sodium salts had a lower ratio of glycerol:P, 1·2:1·0, and gave an atypical Feulgen reaction. The nature of this fraction is discussed later.

Recovery of acidic phospholipids as barium salts from methanol extracts

Ox liver was extracted twice by stirring for 10–15 min. with 1·2 l. of cold acetone/kg. at room temp. after mechanical blending with some of the solvent. The residue was extracted with 2 l. of methanol/kg. at 2° for 24 hr. with occasional shaking. Aqueous 1·5M-$BaCl_2$ was added to the methanol filtrate until no further precipitate formed, and the insoluble barium salts were collected by decantation and centrifuging, and washed with methanol. A measured part of the methanol supernatant, containing the bulk of the lecithin and kephalin, was evaporated at low temperature, and the residue was taken in chloroform–methanol (2:1, v/v) and shaken with water acidified with HCl; the chloroform layer was then washed

several times with water and dried with $CaSO_4$. This fraction (C) was used for preparation of kephalin and lecithin. The crude barium salts were suspended in ether, and the ether-soluble acidic phospholipids (fraction A_1) were recovered by shaking at 2° with a mixture of conc. HCl–methanol–water, 1:4:50, and washing the ethereal solution with water. (Conversion of salts into the free acids in this way was always carried out at 2°, and as rapidly as possible, and the acids were reconverted into the barium or sodium salt for storage overnight at stages.)

The yield of lipid P was about 640–740 mg. of P/kg. of fresh liver, in three different preparations, (L_2, L_3, L_4) of which 17–22% was recovered in fraction A_1. This fraction had a ratio amino N:inositol:P of about 0·5:0·33:1·0, and contained a varied small amount of choline due to lecithin carried down in the barium salts. The content of cardiolipin and other phosphatidic acids was therefore about 15% of A_1, or about 3% of the total lipid P, and the content of phosphatidylinositol about 6–7% of the total.

Fractionation of acidic phospholipids from preparation L_2 *on silicic acid*

The free acid L_2A_1 in ether (2 mg. of P/ml.) was added to 9 vol. of methanol containing 5·0 ml. of 1·5M-$BaCl_2$/l. The insoluble barium salts were washed once with methanol; the ratios amino N:inositol:P were 0·15:0·50:1·0. A portion was converted into the free acid A_2 (46 mg. of P) in ether; this was dried, dissolved in chloroform and immediately chromatographed on 60 g. of silicic acid. After passage of 100 ml. of chloroform and 200 ml. of chloroform–methanol (19:1, v/v) a sharp band I (7 mg. of P) was eluted; this fraction contained no inositol and had a ratio amino N:P of 0·03:1·0; a deacylated sample gave a glycerophosphate and a polyglycerophosphate spot on paper. This band tailed into an intermediate fraction II (5·0 mg. of P) with a ratio inositol:P of 0·5:1·0, and on changing the solvent to chloroform–methanol (9:1, v/v) another sharp band III (26 mg. of P) was eluted; this fraction had ratios amino N:inositol:P, 0·13:0·8:1·0, and a deacylated sample gave glycerophosphorylinositol and glycerophosphorylserine spots on paper. The remaining P (7 mg., ratio amino N:P, 0·60:1·0) was eluted with chloroform–methanol (4:1, v/v).

The front fraction A_2I was evaporated and dissolved in 3·0 ml. of ether; the solution was diluted with 33 ml. of ethanol (to give approximately a concentration of 0·5% of phospholipid) and brought to approx. pH 6·6 with ethanolic 0·2N-NaOH. A flocculent precipitate formed; the mixture was kept at 2°. A similar fraction (6 mg. of P) obtained by chromatographing another portion of A_2 was added to it, and the ethanol-insoluble sodium salts were separated by centrifuging.

The ethanol-insoluble salts (? of phosphatidic acid) were taken up in chloroform and separated from a small residue containing 2·0 mg. of P, of which 1·2 mg. was water-soluble. The solution contained 3·0 mg. of P, with a ratio fatty acid ester:P of 1·0:1·0, and gave an atypical reaction with the Feulgen reagent; a deacylated sample gave 65–70% of the P as phosphomonoester by enzymic assay.

The ethanol-soluble sodium salts (cardiolipin fraction) contained 7·8 mg. of P (i.e. 60% of the fast-running fraction A_2I) with a fatty acid ester:P ratio of 3·4:1·0, indicating the presence of P-free lipid. The solution was evaporated at low temperature and the residue in 2·0 ml. of ether was treated with 6 vol. of acetone at −10°. The acetone supernatant contained 213 mg. of lipid, P, 1·1%. The precipitate weighed 107 mg.; P, 4·2%; fatty acid ester:P, 1·9:1·0; the Feulgen reaction (10 μg. of P) was very faint. A deacylated sample contained less than 5% of phosphomonoester and gave a large spot corresponding to that from cardiolipin.

The low fatty acid ester:P ratio in the ethanol-insoluble salts and the presence of P-free lipid in the ethanol-soluble fraction suggested that some breakdown had occurred.

Phosphatidylinositol. Fraction A_2III (25 mg. of P), obtained by silicic acid chromatography of A_2, was dissolved in 15 ml. of ether and brought to approx. pH 6·6 by addition of ethanolic 0·2N-NaOH; methanol (6 ml.) was added slowly until the solution remained turbid. A semi-crystalline deposit soon formed at room temperature and was collected after several days at 2°; dry wt., 0·51 g.; P, 3·5%; ratios amino N:inositol:P, 0·06:0·90:1·0. The fatty acid composition of this sample of phosphatidylinositol (L_2A_2III) is shown in Table 3. The recovery of inositol in this fraction was equivalent to about 70% of the inositol in L_2A_2.

Separation of phosphatidylinositol and cardiolipin by fractionation of the free acids with ethanol

The crude barium salts from a methanol extract (L_4) of liver were converted into the free acid L_4A_1. A sample (4 μg. of P) chromatographed on silicic acid paper, and stained with Rhodamine G and ninhydrin, showed four spots, corresponding to cardiolipin, phosphatidylethanolamine, phosphatidylserine and phosphatidylinositol. The free acid (840 mg. of P) in 300 ml. of ether was poured with stirring into 2 vol. of methanol containing 15 ml. of 1·5M-$BaCl_2$: the insoluble barium salts were twice taken up in 250 ml. of ether and reprecipitated with 2 vol. of methanol, and then washed with acetone (A_3). The combined supernatants (B) contained mainly kephalin with a little inositide; the serine–ethanolamine ratio was 2·2:1·0.

The further fractionation of A_3 was based on the separation of the ethanol-insoluble acids (phosphatidylinositol) from the ethanol-soluble acids (cardiolipin and phosphatidic acid) and the separation of cardiolipin and phosphatidic acid by fractionation of their sodium salts in ethanol.

The composition of fractions derived from L_4A_1 in the following procedure is shown in Table 1.

The purified barium salts A_3 were converted into the free acid A_4 and treated in dry ether (15 ml.) with 100 ml. of ethanol; the flocculent precipitate was redissolved in 12 ml. of ether and treated with 60 ml. of ethanol. The ethanol-insoluble acids (A_5) were dissolved in ether and the barium salts precipitated by addition of $BaCl_2$ and methanol; phosphatidylinositol was recovered from this fraction (see below). The combined ethanol-soluble acids A_4S were diluted to 300 ml. with ethanol and brought to approx. pH 6·6 with ethanolic 0·2N-NaOH. It was calculated that this volume was sufficient to keep the sodium salt of cardiolipin in solution.

This fractionation gave a substantial recovery of phosphatidylinositol in A_5, and the ethanol-soluble acids were enriched in cardiolipin or other phosphatidic acids, but the carry-over between the fractions was considerable (Table 1).

Fractionation of ethanol-soluble acids as sodium salts

Ethanol-soluble sodium salts. On neutralization of the solution A_4S a precipitate (A_6) formed which was removed after 48 hr. at 2° from the ethanol-soluble sodium salts A_7 (40 mg. of P). A deacylated sample of A_7 gave spots corresponding to polyglycerophosphate and glycerophosphorylserine by paper chromatography, and contained about 3% of phosphomonoester by enzymic assay. The fatty acid ester:P ratio, however, was 2·7:1·0, indicating the presence of P-free lipid. The material was chromatographed at room temperature on silicic acid (50 g.) as the sodium salt in chloroform. Passage of 400 ml. of chloroform removed some P-free lipid (380 mg. containing approx. 1000 μ-moles of fatty acid ester). The phospholipid was then eluted with chloroform–methanol (1:1, v/v) and dissolved in ethanol. The solution was acid and was adjusted to approx. pH 6·6 with ethanolic NaOH, and after 48 hr. at 2° the solution A_7S (35 mg. of P) was decanted from a small deposit. The solution had fatty acid ester:amino N:P ratios, 1·9:0·08:1·0; inositol, not detectable; P, 3·9% on dry wt. On silicic acid paper, spotting 4 μg. of P, it gave a large cardiolipin spot and a small phosphatidylserine spot. The fatty acid composition of this sample of cardiolipin (L_4A_7S) is shown in Table 3.

Ethanol-insoluble sodium salts of ethanol-soluble acids. These salts (A_6) by analysis contained 40% of phosphatides other than phosphatidylinositol and kephalin. A deacylated sample on paper chromatography gave spots corresponding to polyglycerophosphate, glycerophosphorylinositol and glycerophosphorylserine and also a weak glycerophosphate spot. It may be noted that this glycerophosphate spot was produced from material which had not been chromatographed on silicic acid. The material (80 mg. of P) was chromatographed as the sodium salt in chloroform on 65 g. of silicic acid, and the fast-running fraction (35 mg. of P), eluted with chloroform–methanol (94:6, v/v), was dissolved in ether (12 ml.) and ethanol (180 ml.) and adjusted to pH 6·6 with ethanolic NaOH. A precipitate (A_6P) formed immediately and was removed after 48 hr. at 2°. The supernatant material A_6S (20 mg. of P) now recovered as sodium salts soluble in ethanol contained no inositol, and had ratios fatty acid ester:amino N:P of 2·2:0·03:1·0;

Table 1. *Ethanol fractionation of acidic phospholipids*

The fractions were derived from the crude barium salts from the methanol extract L_1 of ox liver, as described in the text. Fraction A_1 contained 22% of the total phospholipid P in L_4.

Fraction	Nature	P in fraction (mg.)	P in fraction (% of total)	Distribution of P (% of total in fraction) Inositide	Kephalin	Other compounds
A_1	Ether-soluble acid from crude barium salts	840	100	33	50	17
A_4	Ether-soluble acid from purified barium salts A_3	520	62	42	32	26
B	Supernatants from A_4	285	34	6	86	8
A_4S	Ethanol-soluble acid from A_4	135	16	30	14	56
A_6	Ethanol-insoluble sodium salts from A_4S	88	10·5	43	17	40
A_7	Ethanol-soluble sodium salts from A_4S	40	4·8	0	11	89
A_6S	Ethanol-soluble sodium salts from silicic acid fraction of A_6	20	2·4	0	5	95
A_6P	Ethanol-insoluble sodium salts from silicic acid fraction of A_6	14	1·7	12	11	77
A_5	Ethanol-insoluble acid from A_4	380	45	48	35	17
A_9	Crystalline inositide from A_5	168	20	90	11	0

a deacylated sample contained less than 3% of phosphomonoester. Chromatography of A_6S on silicic acid paper (4 μg. of P) showed a large cardiolipin spot, faint phosphatidylethanolamine and phosphatidylserine spots, and a faint lipid spot in front of the cardiolipin spot. The fatty acid composition of this cardiolipin (L_4A_6S) is shown in Table 3. The recovery of cardiolipin in A_7 and A_8S was 60 mg. of P. It may be noted that in the presence of impurities the solubility of cardiolipin was low.

'*Phosphatidic acid*' *fraction*. The fraction A_6P, the ethanol-insoluble sodium salts of ethanol-soluble acids, had a fatty acid ester:P ratio of only 1:1, corresponding to a lysophosphatide; ratios amino N:inositol:P, 0·11:0·12:1·0; P, 4·9% on dry wt. A deacylated sample contained 75% of phosphomonoester by enzymic assay. On silicic acid paper, staining with Rhodamine G, A_6P (4 μg. of P) gave weak spots for phosphatidylserine and phosphatidylethanolamine, no cardiolipin spot and a faint spot, similar to that in A_6S, in front of the cardiolipin spot, but these spots obviously only accounted for a small part of the material spotted, and much apparently remained at the origin.

On treatment of a sample of A_6P (10 μg. of P) with 90% acetic acid at 50° and addition of Feulgen reagent a deep-purple colour developed which, unlike that produced by fatty aldehydes, was not extractable into octan-2-ol; an old sample of triolein behaved similarly. A portion of A_6P (216 μmoles of P) was saponified in methanolic 0·2N-KOH 15 min. at 38°; after acidification the fatty material was extracted into ether (wt. 82 mg., equivalent to 290 μmoles of mol.wt. 284) and then refluxed in methanolic 0·8N-HCl to convert fatty acids and any aldehydes into methyl esters and acetals. The product was extracted into ether and washed as usual with $NaHCO_3$ solution and water; wt. 78 mg. This product, which gave a strong Feulgen reaction, was examined by gas chromatography; judging from the area of the peaks, only about 67% was present as volatile substances. These were accounted for as the methyl esters of stearic acid (35%), linoleic acid (44%), oleic acid (10%), linolenic acid (3%), eicosatrienoic acid (1·5%) and small amounts (less than 1%) of C_{13}, C_{14}, C_{15}, C_{16} and C_{17} acids.

It is concluded that the Feulgen reaction given by this fraction is not due to any plasmalogen derivatives, but is analogous to the Kreis reaction with phloroglucinol, and that the fraction, which approximates in composition to a lysophosphatidic acid but contained both nitrogen and inositol, is, in part, a conglomeration of degraded or complexed and 'rancid' phospholipids; these could be derived either from plasmalogens or ester phosphatides, owing partly to the lability towards acid and partly to the auto-oxidation of the highly unsaturated acids present in the inositide and ester phosphatides. The presence of naturally occurring phosphatidic acid in A_6P is of course not excluded, but since, in view of the anomalous reactions and the suspicion of breakdown, it appeared impossible to conclude that any phosphatidic acid positively identified was in fact natural, no attempt was made at further fractionation. The amount of P recovered in A_6P was 1·7% of the P in the crude acidic phospholipid A_1 and 0·4% of the total lipid P.

Phosphatidylinositol. The ethanol-insoluble acids A_5 (320 mg. of P) were recovered as barium salts insoluble in methanol and reprecipitated from ether solution with methanol. The methanolic supernatant contained 102 mg. of P; ratio amino N:P, 0·8:1·0. The barium salts were converted into the sodium salts (180 mg. of P) insoluble in ethanol; these were dissolved in ether (100 ml.) and the solution was treated with methanol until turbid; the semi-crystalline deposit, A_9, contained 168 mg. of P; ratio amino N:inositol:P, 0·1:0·9:1·0; chromatography on silicic acid paper gave a large phosphatidylinositol and weak phosphatidylethanolamine and phosphatidylserine spots. The fatty acid composition (L_4A_9) is shown in Table 3. The recovery of phosphatidylinositol in L_4A_9, based on inositol assays, was equivalent to 64% of the inositide in L_4A_1.

Separation of kephalin and lecithin from the methanol-soluble fraction C *of the extract* L_3

A portion (79 mg. of P) of fraction L_3C, recovered after removal of the methanol-insoluble barium salts, containing the ester phosphatides and about 3% of sphingomyelin by assay of alkali-stable P, was chromatographed on 75 g. of silicic acid; after passage of 200 ml. of chloroform, which eluted a little P-free lipid, the solvent was changed successively to chloroform–methanol (9:1, 4:1 and 1:1, v/v), with which 90% of the ester phosphatides was eluted; the tail of lecithin and sphingomyelin was discarded. The eluate fractions were pooled, on the basis of P, amino N and fatty acid ratios, as shown in Table 2, the lecithin eluates being pooled in four fractions with decreasing iodine values. The kephalin and the first three lecithin fractions, which contained small amounts of plasmalogens, were treated individually with 90% acetic acid at 38°, and the free aldehyde, lysophosphatide and diacyl phosphatide were separated chromatographically by Dr G. M. Gray; the nature of the aldehydes and the mean fatty acid composition of the kephalin and lecithin have been reported (Gray, 1960). The fatty acid composition of the plasmal-free lecithins separated from the first three lecithin fractions is shown in Table 3.

Table 2. *Fractionation of liver ester phosphatides* L_3C *on silicic acid*

The starting material was the ester-phosphatide fraction C from a methanol extract L_3 after removal of insoluble barium salts of acidic phospholipids.

Eluate nos.	Fraction	Per cent of total P recovered	Molar ratio aldehyde:P (P = 100)	Double bonds/mol. of P
14–36	Kephalin	16	18	2·0
37–54	Lysokephalin	4	—	—
	Lecithin	5	—	—
55–67	Lecithin I	30	5	4·5
68–79	Lecithin II	32	11	3·0
80–91	Lecithin III	10	12	2·1
92–94	Lecithin IV	3	24	1·9

Table 3. *Fatty acid composition of phospholipid fractions from ox liver*

Gas chromatography of methyl esters on Reoplex 400 or Apiezon L. Values are expressed as percentage of the total methyl esters. tr., Trace.

		Cardiolipin		Phosphatidylinositol		Lecithin L_3C		
Acids	Designation	L_4A_7S	L_4A_6S	L_2A_2 III	L_4A_8	I	II	III
C_{10}–C_{15}	—	—	0·8	0·5	—	tr.	0·2	2·4
Palmitic	16:0	0·9	1·7	1·9	1·9	8·3	15·3	18·7
Palmitoleic	16:1	1·3	1·5	—	0·3	—	0·6	2·2
Heptadecanoic	17:0	1·2	—	0·9	0·6	0·5	0·8	0·8
—	17:?	—	0·4	0·2	—	0·4	0·7	1·1
Stearic	18:0	8·4	1·8	54·6	46·8	39·6	35·0	25·6
Oleic	18:1	5·7	8·1	5·4	10·2	3·7	19·5	31·0
*iso*Oleic	*iso* 18:1	—	—	2·6	—	3·7	—	—
Linoleic	18:2	63·0	70·0	3·5	8·6	2·9	11·3	11·6
Linolenic	18:3	12·0	15·5		2·5	tr.	tr.	—
Nonadecanoic	19:0	—	—	—	0·5	—	—	—
(Unidentified)	?	—	—	—	2·5	—	—	—
(Unidentified)	?	—	—	—	1·9	—	—	—
Eicosatrienoic	20:3	2·4	—	8·2	6·0	5·2	2·7	1·0
Arachidonic	20:4	2·8	—	16·8	7·1	11·4	5·3	3·2
Eicosapentaenoic	20:5	2·0	—	—	4·3	3·8	3·0	
Docosapentaenoic	22:5	—	—	5·2	7·1	17·5	5·1	1·4
Docosahexaenoic	22:6	—	—	—	—	2·9	0·3	0·8

DISCUSSION

Faure *et al.* (1959) found that the polyglycerophosphatide from ox liver was identical with cardiolipin from heart muscle in general composition and serological activity. The chromatographic properties of the polyglycerophosphatide isolated in the present work, and the structure of the water-soluble ester prepared from it, confirm this identification. The discrepancies in earlier analyses (McKibbin & Taylor, 1952; Dawson, 1958; Marinetti, Erbland, Albrecht & Stotz, 1957) probably arise from the fact that the cardiolipin fraction obtained chromatographically is liable to be contaminated on the one hand with P-free lipid and on the other with degraded phosphatides. The Feulgen reaction given by crude preparations is attributable to rancidity rather than to the presence of plasmalogen derivatives, and it occurred whether the fractionation was carried out by chromatography or by solvent fractionation of the free acids. Cardiolipin and phosphatidylinositol as well as the plasmalogens and ester phosphatides were substantially recovered from silicic acid columns, though prompt neutralization of acid solutions is desirable, but the possibility that some breakdown occurs either on columns or during solvent fractionation cannot be excluded. Because the proportion of cardiolipin is small the rancid or degraded phosphatide contaminating this fraction is possibly more noticeable in extracts of liver than from other tissues; it amounted at most to 0·4% of the total P, which is perhaps surprisingly low, considering the high degree of unsaturation of the liver phosphatides. Its accumulation in the fraction in which phosphatidic acid might be expected obviously complicates the unequivocal isolation of naturally occurring phosphatidic acid. The occurrence of small amounts of P-free lipid is probably another sign of breakdown. No evidence, however, was obtained of the presence of any substantial amount of phosphatidic acid or unidentified phospholipids in the early stages of fractionation.

In summary, cardiolipin has been isolated from ox liver by Faure *et al.*(1959) and, in the present work, in yields of 1–2% of the total phospholipid P; it has also been identified in rat liver (Macfarlane, Gray & Wheeldon, 1960) and in sheep liver (Dawson, 1960) in amounts approx. 3–4% of the total phospholipid. It is eluted from silicic acid columns with low concentrations of methanol in chloroform. The apparent failure of Hübscher & Clark (1960) to find any cardiolipin in ox or rat liver, fractionated by this method, and their success in readily isolating phosphatidic acid with 80–90% of unsaturated acids in yields of 0·8 and 1·5% of the total phospholipid, when other workers (e.g. Hokin & Hokin, 1958; Dawson, 1954, 1960; Macfarlane *et al.* 1960) have found phosphatidic acid in tissues only in trace amounts, if at all, call for some comments on their criteria of identification. (1) No evidence was offered, e.g. by paper chromatography of the intact phospholipid, that the nitrogen-free component was homogeneous and different from cardiolipin. (2) The main analytical difference between phosphatidic acid and cardiolipin is the ratio of glycerol to phosphorus. Hübscher & Clark (1960) apparently determined glycerol by periodate oxidation and estimation of formaldehyde after acid hydrolysis of the phospholipid. On acid hydrolysis cardiolipin gives firstly a high proportion of glycerodiphosphate (Gray & Macfarlane, 1958; Macfarlane & Wheeldon, 1959), which is not oxidized by periodate and only slowly hydrolysed in acid. No evidence is given that the conversion into orthophosphate and therefore the liberation of glycerol were complete. (3) The identification of 'glycerophosphate' formed from the phospholipid by mild alkaline hydrolysis was not rigorous, and no evidence was offered that the 'glycerophosphate' was distinct from the polyglycerophosphate derived from cardiolipin.

It should be emphasized that this criticism is not intended as an argument against the presence of naturally occurring phosphatidic acid, for if this substance is an intermediate it is very likely to be present. But the fact that Hübscher & Clark (1960) apparently did not find cardiolipin in the liver and have not, in the writer's opinion, excluded the presence of cardiolipin in their 'phosphatidic acid' fraction, together with the similarity of the fatty acid composition to that of cardiolipin, calls for some caution in accepting the isolated phosphatidic acid as the naturally occurring intermediate. As the authors themselves point out, it is difficult to reconcile the fatty acid composition with that of an intermediate in the biosynthesis of kephalin, lecithin or inositides, in which, generally speaking, 50% of the fatty acids are saturated.

In considering the fatty acid composition of the cardiolipin and phosphatidylinositol isolated in the present work it should be noted that the degree of purity of the samples analysed was sacrificed in favour of a larger yield; the presence and effect of kephalin derivatives, the most usual impurity, can be assessed. The possibility that the apparent occurrence of stearic acid as the sole saturated acid in phosphatidylinositol might be due to selective isolation of a sub-family was mentioned previously (Gray & Macfarlane, 1958); the yield was here increased to 64–70% with 90% purity, and stearic acid is still clearly the predominant saturated acid; in samples from different animals the relative proportions of the different polyenoic acids however varied appreciably. The presence of 8% of stearic acid and polyenoic acids in the cardiolipin sample L_4A_7S can be correlated with the presence of 10% of kephalin indicated by the amino-N content. The predominant acid, as in cardiolipin from heart muscle, is linoleic acid, but linolenic acid was present in amounts (12–15%) considerably greater than were found in any other phospholipid.

The nature and structure of the unsaturated acids present in ox-liver ester phosphatides were established by Klenk and his colleagues (Klenk & Dreike, 1955; Klenk & Tomuschat, 1957). The mean composition of the ox-liver kephalin and lecithin fractions analysed by gas chromatography (Gray, 1960) correspond closely, qualitatively and quantitatively, with their findings; in this species, in contrast with the rat (Macfarlane *et al.* 1960), the C_{22} pentaenoic acid preponderates over the C_{22} hexaenoic acid.

The successive lecithin fractions obtained by chromatography on silicic acid (Table 3) had decreasing iodine values, due to increasing proportions of linoleic acid and oleic acid in place of higher polyenoic acids (cf. Rhodes & Lea, 1956). All the fractions contained the same proportion of saturated acids, with a higher proportion of stearic acid, particularly in the initial fraction eluted (I). Chromatographic fractionation of liver lecithin and hydrolysis with *Clostridium welchii* lecithinase could afford a useful range of diglycerides for experiments in biosynthesis.

SUMMARY

1. The polyglycerophosphatide of ox liver isolated by chromatography on silicic acid and by solvent fractionation of barium salts was identified as cardiolipin by the similarity in chromatographic and chemical properties of the phospholipids and of the phosphoric esters derived from them by mild alkaline hydrolysis.

2. The Feulgen reaction given by crude cardiolipin fractions is attributed to the presence of

rancid phosphatides, whose amount is small in relation to the total phospholipid.

3. Degraded phosphatides were present in the fraction expected to contain phosphatidic acid. It was therefore considered impossible either to exclude or confirm the presence of naturally occurring phosphatidic acid.

4. The bearing of these facts on the isolation of phosphatidic acid reported by Hübscher & Clark (1960) is discussed.

5. Linoleic acid (70%) and linolenic acid (12–15%) were the main fatty acids in ox-liver cardiolipin.

6. Phosphatidylinositol isolated in yields of 64–70% of the total inositide contained stearic acid as virtually the only saturated acid (45–55%) and polyenoic acids in varying proportion as the main unsaturated components.

7. Successive fractions of liver lecithin with decreasing iodine values obtained by chromatography on silicic acid contained similar proportions of saturated acids and decreasing proportions of polyenoic acids.

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The Amide Nitrogen Content of Gelatins

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It has long been known that the isoionic points of gelatins prepared from alkali-treated collagens are in general considerably lower than those of gelatins extracted after acid pretreatment. Ames (1952) has shown that this fall in the isoionic point of alkali-processed gelatins is associated with a loss of ammonia during pretreatment. The ammonia has been assumed to be derived mainly from hydrolysis of amide side chains which thus give rise to new free carboxyl groups in the intact molecule of the gelatin precursor. Even acid pretreatment may result in some loss of amide groups, if conditions are sufficiently severe (Ames, 1957). A picture has gradually emerged of a series of possible gelatins, ranging from those with an isoionic point above pH 9, with substantially all the original amide groups still intact, to those prepared after a prolonged lime soak with a limiting minimum isoionic point at approximately pH 4·80 and presumably zero amide content. The exact relationship between the isoionic point and the amide content has not, however, been experimentally investigated. A survey of these two characteristics for a representative selection from a wide range of gelatins available in this Laboratory is described in this paper. For this purpose a rapid and accurate method for the determination of amide nitrogen in gelatin was developed.

EXPERIMENTAL

Gelatins. These were selected from about 150 samples to represent, as far as possible, a continuous range of isoionic points (p*I*) between 4·8 and 9·5. One sample only was available in the range p*I* 5·5–7·5. To remove ammonium salts, possibly present in some gelatins, 10% solutions were deionized with mixed-bed ion-exchange resins (Janus, Kenchington & Ward, 1951). The solutions were allowed to set and the gels dried in a current of air at approximately 20°.

Methods

Moisture. The loss of weight on drying a thin film of gelatin for 18 hr. at 105° was used to calculate moisture content (B.S. 757: 1959 and Eastoe & Williams, 1959).

Ash. The gelatin was slowly incinerated in a platinum dish over a small Bunsen flame and the residue was heated for 2 hr. at 550° in an electric muffle furnace. The small fluffy pellets of ash were transferred with a needle to a piece of foil and weighed on a microbalance.

Isoionic point. This was determined by the deionization method of Janus, Kenchington & Ward (1951) with a glass electrode and Cambridge pH meter.

Determination of amide nitrogen

0·05 M-*Sodium tetraborate*–0·15 N-*sodium hydroxide.* Sodium tetraborate (19·1 g. of $Na_2B_4O_7,10H_2O$, A.R.) and NaOH (6 g., A.R.) were dissolved in 1 l. of water, previously boiled to remove CO_2.

1% (*w*/*v*) *Boric acid with indicator.* Boric acid (10 g. of H_3BO_3, A.R.) was dissolved in 1 l. of boiled water and 8 ml. of mixed indicator solution was added. The cold solution was adjusted to a neutral-grey colour by addition of approximately 0·7 ml. of 0·1 N-NaOH. This reagent kept well in borosilicate glass in the dark.

Mixed indicator. Equal volumes of ethanolic 0·033% methylene blue and 0·05% methyl red solutions were mixed.

Procedure. Approximately 2·5 g. of gelatin was accurately weighed into a 500 ml. round-bottom flask and dissolved in 100 ml. of warm 2 N-HCl. A few alundum chips (no. 14 grit, Norton Abrasives Ltd.) were added and the solution was boiled under reflux for 1 hr. Sufficient mixed indicator was added to the cold solution to enable the colour to be easily seen. Some gelatins gave rise to dark-brown hydrolysates and for these it was necessary to use external indicator paper. The flask was stoppered and 2 N-NaOH was added initially in approximately 10 ml. portions and later in smaller amounts, the stopper being removed only during the additions. The contents were mixed and kept cool by partially immersing the flask in cold water. The pH of the solution was adjusted to the end point of the indicator (approx. pH 5·2) and the solution diluted to 250 ml.

The steam-distillation apparatus of Yuen & Pollard (1953) was used for separation of ammonia. Water containing 0·1% of syrupy phosphoric acid was added to the steam-generator flask and approximately 100 ml. was boiled off to remove CO_2 before a series of distillations. A portion (20 ml.) of the neutralized hydrolysate was added to the distillation apparatus, followed by 20 ml. of 0·05 M-sodium tetraborate–0·15 N-NaOH solution. The distillate was collected in 10 ml. of the 1% boric acid solution, distillation being carried out until approximately 40 ml. had collected (approximately 5 min.). When necessary, frothing was prevented by the initial addition of 2 drops of silicone M.S. experimental antifoam emulsion (Hopkin and Williams Ltd.). The distillate was titrated with 0·01 N-HCl to the first faint permanent pink colour. Blanks (without gelatin) were carried through the whole procedure and the blank titration was subtracted from the experimental values. The amide-nitrogen value was calculated for the dry, ash-free protein.

Factors affecting the accuracy of the amide determination

Solution for absorbing ammonia. Plain aqueous 1% boric acid, widely used for ammonia absorption in determinations of total N, is normally slightly on the acid side of the achromatic end point of screened methyl red indicator. The requirements for ammonia absorption and titration are identical in amide-N and total-N determinations, except that, under some conditions CO_2 may distil over in amide determinations, owing to the lower pH of the boiling solution. In blank determinations the amount of CO_2 may exceed the quantity of ammonia distilling. If the solution is initially on the acid side of the end point, distillation of CO_2 does not result in a colour change and may not be noticed. A convenient reagent is obtained by adding a definite concentration of indicator to the boric acid solution in bulk and then adjusting it to the end point.

Conditions for distillation of ammonia. In the present investigation acid hydrolysates were made alkaline in two stages. 2 N-NaOH solution was first added to bring the solution to approximately pH 5·0. This step was not critical in producing variations in results, if carried out as described. A portion of the diluted hydrolysate at pH 5·0 was then mixed with an equal volume of an alkaline solution in the distillation apparatus. The use of 0·05 M-sodium tetraborate, 0·05 M-sodium tetraborate–0·15 N-NaOH and N-NaOH gave final solutions of pH 8·6, 9·95 and > 13 respectively at 22°.

N-NaOH solution was unsuitable because it brought about continuous loss of ammonia from boiling gelatin hydrolysates, presumably owing to decomposition of amino acids at the high pH. Neither 0·05 M-sodium tetraborate nor 0·05 M-sodium tetraborate–0·15 N-NaOH did this. Experiments showed that distillation of ammonia from a standard (approx. 0·004 M) NH_4Cl solution was complete when the first 20 ml. (of the specified 50 ml.) of distillate had collected, irrespective of whether 0·05 M-sodium tetraborate or N-NaOH was added.

Experiments to investigate apparent recovery of ammonia from the standard NH_4Cl solution showed that it was variable and up to 1% lower with 0·05 M-sodium tetraborate than with N-NaOH, which gave the theoretical recovery. The end point was also less sharp with sodium tetraborate. These effects were traced to distillation of CO_2 at the lower pH. The higher pH value reached with the 0·05 M-sodium tetraborate–0·15 N-NaOH reagent greatly reduced the quantity of CO_2 distilling over and largely overcame the difficulty.

Distillates from 20 ml. portions of NH_4Cl solution, to which equal volumes of the various alkaline reagents had been added, were titrated to a faint-pink colour, boiled for 30 sec. to remove CO_2 and quickly cooled. Where CO_2 was present the solution became green again and more 0·01 N-HCl was added to restore the pink colour. The initial and

Table 1. *Mean titration values for distillates from 20 ml. of 0·00398 M-ammonium chloride solution made alkaline with various reagents*

7·95 ml. corresponds to a theoretically complete recovery.

Reagent		0·01 N-HCl added (ml.) Initially	Total after boiling
0·05 M-Sodium tetraborate	Blank	0·00	0·13
	NH_4Cl	7·90	8·08
	Difference	**7·90**	**7·95**
0·05 M-Sodium tetraborate–0·15 N-NaOH	Blank	0·11	0·14
	NH_4Cl	8·04	8·09
	Difference	**7·93**	**7·95**
N-NaOH	Blank	0·16	0·16
	NH_4Cl	8·11	8·11
	Difference	**7·95**	**7·95**

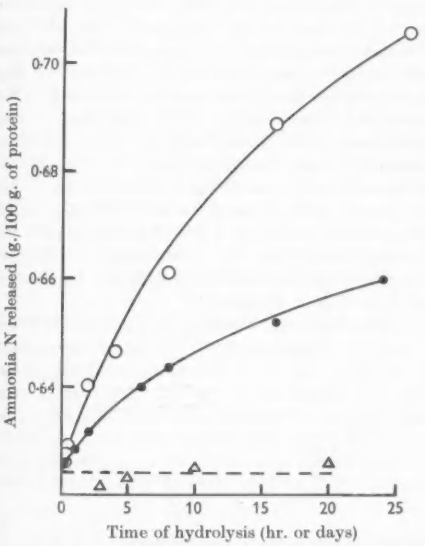

Fig. 1. Ammonia N released from acid-processed gelatin after hydrolysis with hydrochloric acid for various periods. ○, Boiling 6 N-HCl; ●, boiling 2 N-HCl; △, 12 N-HCl at 37°. Time is expressed in hours for 2 N- and 6 N-HCl and in days for 12 N-HCl.

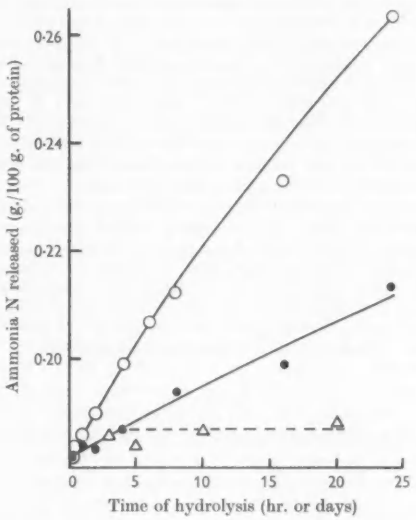

Fig. 2. Ammonia N released from lime-processed gelatin after hydrolysis with hydrochloric acid for various periods. Details of symbols and time scale are the same as those given in Fig. 1.

total volumes of acid added are given in Table 1. Removal of CO_2 increased the titration by 0·13–0·18 ml. of 0·01 N-HCl with 0·05 M-sodium tetraborate and by only 0·03–0·05 ml. with 0·05 M-sodium tetraborate–0·15 N-NaOH. The distillate from the solution with N-NaOH remained pink after boiling. The differences between the initial titrations for NH_4Cl solution and blank with 0·05 M-sodium tetraborate and 0·05 M-sodium tetraborate–0·15 N-NaOH were respectively 0·05 and 0·02 ml. lower than with N-NaOH (Table 1). Boiling the distillate enabled identical results to be obtained with all three reagents but was not adopted because it complicated the experimental procedure. Instead, the sodium tetraborate–NaOH reagent was used since it resulted in titration values only 0·3% below theoretical and gave much sharper end points than those with sodium tetraborate alone.

Hydrolysis of the amide groups of gelatin by hydrochloric acid. The release of ammonia from two gelatins (one an acid-processed sample of high amide content and the other lime-processed and of fairly low amide content) by boiling 2 N-HCl and boiling 6 N-HCl for periods from 15 min. to 24 hr. and by 12 N-HCl at 37° for 3–20 days was studied. The ammonia was determined in the 2 N-HCl hydrolysates as described above. The 6 N-HCl hydrolysates were initially

neutralized with 6N-NaOH and the bulk of the acid from the 12N-HCl hydrolysates was removed by evaporation on a warm plate at 35° before evaporation of the last portion *in vacuo* at room temperature. The ammonia was then determined.

The quantities of ammonia N released in these experiments are summarized in Figs. 1 and 2. With 12N-HCl, a very slight increase for the amide value of the acid-processed gelatin was evident over the period 3–20 days. The scatter of individual values, however, was of the same order as this increase. No such increase was evident for the lime-processed gelatin. Mean values of 0·624 and 0·187% respectively were calculated and are represented by the horizontal lines in Figs. 1 and 2.

Both boiling 2N-HCl and 6N-HCl showed substantial increases in the quantity of ammonia liberated in periods up to 24 hr. Almost equal amounts of excess of nitrogen were liberated in the period from 15 min. to 24 hr. from both types of gelatin, 0·032% by 2N-HCl and 0·078% by 6N-HCl. The quantity of ammonia N released during short periods of heating ($\frac{1}{4}$–1 hr.) were all closely similar for both acid concentrations and consequently did not differ from values extrapolated to zero time by more than the experimental error. For the acid-processed gelatin, extrapolation gave values of 0·624% with both 2N-HCl and 6N-HCl, which was identical with the mean value with 12N-HCl. For the lime-processed gelatin, identical values of 0·182% were obtained by extrapolation, slightly lower than the mean value of 0·187% obtained with 12N-HCl at 37°.

Since all three methods of hydrolysis gave closely similar results for both types of gelatin studied, 2N-HCl was selected for routine use, being more convenient than 12N-HCl and having a lower rate of release of additional ammonia than 6N-HCl. Extrapolation to zero time did not result in values significantly different from those obtained with heating periods up to 1 hr. with 2N-HCl. A period of 1 hr. was selected, since it provides a safety margin.

RESULTS

The amide-nitrogen contents of a series of deionized gelatins are summarized in Table 2 in order of increasing isoionic points. The amide values were determined by the method described in the Experimental section and also after hydrolysis with 6N-HCl for 2 hr., a small correction, based on the average of the initial slopes of the upper curves in Figs. 1 and 2, being applied for additional non-amide nitrogen set free during this period. Values obtained by the two methods showed good agreement, being slightly higher with 6N-HCl.

DISCUSSION

The method for amide nitrogen described has an accuracy approaching that of the lengthier procedure of Gordon, Martin & Synge (1941). Errors due to distillation of carbon dioxide have been reduced to the same order as those associated with the normal titration end point. The findings of Leach & Parkhill (1955) that identical values for the amide content of wool are obtained after hydrolysis with either hot 2N-hydrochloric acid for 1 hr. or 12N-hydrochloric acid at 37° for 10 days have been confirmed for gelatin. Tristram (1949) mentioned that Rees (1946) also found that hydrolysis with dilute boiling acid for 2 hr. invariably gave the same results as 10N-hydrochloric acid at 37° for 10 days; it seems likely that the present method would be valid for all proteins that dissolve readily in 2N-hydrochloric acid. For those not easily soluble, a preliminary treatment with more concentrated acid, followed by dilution before boiling, is suggested.

It has been found that approximately 0·078% of ammonia nitrogen is slowly liberated from gelatin on boiling with 6N-hydrochloric acid for 24 hr., after the rapid initial release of ammonia from amide groups. This experimental value is in good agreement with the value (0·0764%) calculated from the serine and threonine contents of gelatin (Eastoe, 1955) and the data of Rees (1946) for the

Table 2. *Amide-nitrogen contents and isoionic points of deionized gelatins*

Designation	Type	Moisture (%)	Ash (%)	p*I*	Amide N (g./100 g. of protein) 2N-HCl (1 hr.)	Amide N (g./100 g. of protein) 6N-HCl (2 hr.)
133	Limed	15·6	0·064	4·80	0·109	0·132
E.K.	Calf skin, limed	13·5	0·03	4·82	0·018	0·027
70	Sinew, lime–NaOH	16·3	0·083	4·82	0·054	0·063
90	Hide, limed	15·1	0·032	4·90	0·095	0·103
1	Hide, limed	15·8	0·092	4·94	0·111	0·118
150	Hide, limed	—	—	4·96	0·178	—
19	Calf skin, lime–NaOH	14·7	0·015	5·15	0·200	0·212
126	Bone glue	15·4	0·13	5·47	0·493	0·508
8790	—	16·1	0·073	6·5	0·442	0·451
A.S.	Ox sinew, acid	16·6	0·34	7·5	0·613	0·634
142	Ossein, acid	16·9	0·037	7·8	0·498	0·511
136	Pig skin, acid	16·9	0·021	8·4	0·543	0·555
96	Pig skin, acid	16·5	0·018	9·4	0·624	0·628
149	Pig skin, acid	16·6	0·016	9·4	—	0·615

decomposition of these amino acids in boiling 6N-hydrochloric acid.

The value of 0·62% of amide nitrogen found for the two pig-skin gelatins of p*I* 9·4 is identical with that determined previously for a similar gelatin by resin chromatography (Eastoe, 1955). Bowes, Elliott & Moss (1955) found 0·52 and 0·66% respectively for citrate-soluble collagen and native ox-hide collagen. The intermediate value for acid-processed pig-skin gelatin suggests that practically all the amide groups have remained intact during pretreatment and extraction.

The points plotted in Fig. 3, with two conspicuous exceptions, lie close to the smooth curve represented by the continuous line. The exceptional samples are the acid sinew gelatin (A.S.) and the bone glue (126). These samples and a long-limed gelatin of European origin differed from all the other gelatins in that their solutions in both 2N-hydrochloric acid and 6N-hydrochloric acid rapidly darkened on heating and deposited a brown precipitate, the hydrolysates developing a marked caramel odour. This behaviour was attributed to the presence of polysaccharide impurities, the component sugars of which were released by hydrolysis and reacted with amino compounds from the gelatin to produce brown products and free ammonia (browning reaction, see Blake & Plaster, 1950). Similar amounts of ammonia were produced with both concentrations of acid.

The smooth curve on which the remaining points lay was similar in shape to the titration curve of an alkali-processed gelatin of p*I* 4·92 (Kenchington & Ward, 1954), which is shown by a broken line in Fig. 3. The titration curve runs approximately parallel to that relating amide content to isoionic point. The dotted line in Fig. 3 was obtained by displacing the titration curve upwards by approximately 0·04 m-equiv/g., so that it crosses the *X*-axis at p*I* 4·80. This derived curve is intended to correspond to that of a gelatin the amide groups of which have been completely removed by alkaline pretreatment. It is similar in shape and position to the amide–p*I* curve, except that it is slightly less steep below pH 5 and rises much more steeply above pH 8. The deviation of the curves at high pH has not been explained. The amide–p*I* curve depends upon values for only two gelatins of high isoionic point, which, however, agree closely with one another.

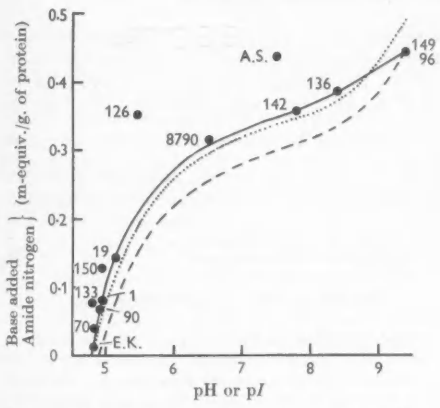

Fig. 3. Relation between amide-N content and isoionic point for a range of gelatins compared with the titration curve for an alkali-processed gelatin. Amide N–p*I* curve (full line); titration curve for gelatin of p*I* 4·92 (Kenchington & Ward, 1954) (broken line); titration curve moved upwards by 0·04 m-equiv./g. (dotted line). Designation of gelatins is as given in Table 2.

The relative shapes of the portions of the curves below pH 8 may be theoretically justified by considering the gradual replacement of carboxyl groups in an isoionic gelatin of zero amide content by amide groups. Wherever a given number of charged carboxyl groups are replaced by electrically neutral amide groups, there will be a shift in isoionic point equal to the shift in pH produced by removing the same number of protons from the gelatin molecule during titration. Amide groups replacing uncharged carboxyl groups, however, will not affect the isoionic point. There will be a preponderance of charged over uncharged carboxyl groups in the pH range shown in Fig. 3, and the proportion of uncharged carboxyl groups will fall rapidly with increase in pH above pH 4·8. Thus, near pH 4·8, a significant but not predominant proportion of amide groups introduced would replace uncharged carboxyl groups and so would not contribute to a change in isoionic point. Because of the extra number of amide groups needed to replace uncharged carboxyl groups the amide–p*I* curve would be expected to be steeper than the titration curve in this region. At higher pH values the number of amide groups replacing uncharged carboxyl groups will tend towards zero so that the two curves will have the same shape.

SUMMARY

1. A rapid and convenient method for the determination of amide nitrogen in gelatin, based on preliminary hydrolysis with 2N-hydrochloric acid, is described.

2. No significant decomposition, resulting in release of non-amide ammonia, occurs during the distillation stage. Errors resulting from the distillation of carbon dioxide have been decreased to a low level. The method of hydrolysis gives

results very close to those obtained with 12N-hydrochloric acid at 37°.

3. The amide contents of a series of deionized gelatins, ranging from p*I* 4·80 to 9·4, are related to their isoionic points by a curve of similar shape to the titration curve.

4. Certain gelatins and glues that show evidence of containing polysaccharide impurities give rise to larger quantities of ammonia on hydrolysis than would be expected from their isoionic points.

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Formation of Labelled Amino Acids by Exchange Transamination

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Glutamate–aspartate transaminase, which catalyses reaction (1)

$$\text{Glutamate} + \text{oxaloacetate} \rightleftharpoons \text{aspartate} + \alpha\text{-oxoglutarate} \quad (1)$$

is known to catalyse also the exchange of an amino group (reaction 2) between glutamate and its keto acid analogue (Nisonoff, Barnes, Enns & Schuching 1954; Jenkins, Yphantis & Sizer, 1959).

$$[^{14}\text{C}]\text{Glutamate} + \alpha\text{-oxoglutarate} \rightleftharpoons \text{glutamate} + \alpha\text{-}[^{14}\text{C}]\text{oxoglutarate}. \quad (2)$$

To the best of our knowledge the possible catalysis of the corresponding reaction (3), between aspartate and its keto acid analogue,

$$\text{Aspartate} + [^{14}\text{C}]\text{oxaloacetate} \rightleftharpoons [^{14}\text{C}]\text{aspartate} + \text{oxaloacetate} \quad (3)$$

has not previously been investigated.

If such reactions occurred *in vivo* they would have a bearing on the interpretation of the results of $^{14}CO_2$-feeding experiments. Accumulation of label in glutamate or aspartate would then not necessarily be indicative of synthesis in the usual sense, since these acids could be formed (in reactions 2 and 3) at the expense of their unlabelled counterparts. Clearly, however, much would depend upon the relative rates of the normal transamination and what we shall call 'exchange transamination' (reactions 2 and 3). The following experiments were therefore primarily carried out to determine (*a*) whether or not $^{14}CO_2$ could be introduced into aspartate in a model system by carboxylation of phosphopyruvate followed by exchange transamination of the resulting oxaloacetate, and (*b*) if the rate of incorporation was of the same order as that of the corresponding system in which glutamate serves as the amino donor in the normal transamination.

MATERIALS AND METHODS

Phosphoenolpyruvate was from the Sigma Chemical Co., St Louis, Mo., U.S.A. L-[^{14}C]Glutamic acid and sodium [^{14}C]bicarbonate were obtained from The Radiochemical Centre, Amersham, Bucks. Castor beans were kindly given by the Baker Castor Oil Co., San Diego, Calif., U.S.A.

Enzymes. Mitochondria were prepared from the endosperm of 50 4-day castor-oil-bean seedlings (*Ricinus communis* var. Cimarron) according to the method of Beevers & Walker (1956) and washed three times in cold 0·5M-sucrose–0·1M-phosphate (NaH_2PO_4) adjusted to pH 7·1 by the addition of potassium hydroxide. They were then sus-

pended in 200 ml. of ice-cold water and left to stand in the cold for 30 min. After centrifuging at 33 000 *g* for 30 min. at 0°, spectrophotometric assay (Walker & Ranson, 1958) indicated that almost all the glutamate–aspartate transaminase was present in the supernatant. Two volumes of ice-cold saturated $(NH_4)_2SO_4$ were added to the supernatant and the mixture was left to stand in the cold room overnight. The precipitate which formed was removed (by centrifuging at 33 000 *g* at 0° for 10 min.) and stored in the deep-freeze in saturated $(NH_4)_2SO_4$ at −20°. For use the precipitate was again separated by centrifuging, redissolved in 10 ml. of 0·1% 2-amino-2-hydroxymethyl-propane-1:3-diol (tris) buffer, pH 7·4, and dialysed in the cold for 72 hr. against 15 l. (3 × 5 l.) of the same buffer.

Cell-free extracts of *Kalanchoë* leaves were prepared as described by Walker (1957). The fraction of protein (150 mg.), which was precipitated between 30 and 60% saturation with $(NH_4)_2SO_4$, was redissolved in 25 ml. of tris buffer, pH 7·4, and dialysed in the cold for 72 hr. against the same buffer before use.

Chromatography. Samples were chromatographed in one dimension on Whatman no. 1 paper with butanol–acetic acid–water (74:19:50) as the developing solution.

Degradation of aspartate. The reaction mixture consisted of 2 ml. of eluted aspartate + added carrier (3·0 μmoles of aspartate), 0·2 ml. of 3 M-acetate buffer, pH 4·9, and 0·1 ml. of pyruvate solution (made by dissolving 0·66 g. of sodium pyruvate in 10 ml. of 0·2 M-acetate buffer, pH 4·9). After equilibration, 0·6 ml. of aspartate decarboxylase prepared from *Nocardia* (Crawford, 1958) was added. The vessel was incubated for 1½ hr. at 22° and was continually flushed with air. Residual radioactivity was counted with a thin end-window Geiger–Müller tube on aluminium planchets covered with lens tissue.

RESULTS

Introduction of $^{14}CO_2$ into aspartate

In a preliminary experiment, *Kalanchoë* extract was incubated with sodium [^{14}C]bicarbonate and phosphoenolpyruvate together with the additions shown in Table 1. Samples were then chromatographed and radioautographs showed that more than 99% of the activity was restricted to an amino acid with the same R_F as aspartate. The actual level of $^{14}CO_2$ fixed was measured with a Geiger–Müller end-window counter placed directly on the chromatograms and the values obtained are given in Table 1.

The small fixation in the absence of added amino acids is possibly attributable to the presence of traces of free amino acids in the enzyme solution. Evidently the fixation in the presence of added amino acids was approaching completion, if equilibrium had not actually been achieved, with the major part of the added $^{14}CO_2$ incorporated into aspartate. At this stage the fixation with aspartate as the amino donor (86%) was not greatly different from that with glutamate as the amino donor (83%).

Rate measurements of fixation of carbon dioxide

To obtain comparative measurements of the actual rates of incorporation of $^{14}CO_2$ into aspartate further experiments were carried out in which samples were taken at frequent time intervals (Fig. 1). In the experiment shown in Fig. 1 the initial rate with aspartate as the amino donor was approximately three times that with glutamate as the amino donor. In each of two additional experiments (with a second sample of enzyme) in which 2 μmoles and 1 μmole of amino donor were added this rate was again approximately threefold (Fig. 2).

The radioative amino acid which accumulated in the reaction mixtures to which aspartate had been added was eluted from the chromatograms and incubated with a cell-free extract of *Nocardia*. This preparation catalyses specifically the β-decarboxylation of L-aspartate (Crawford, 1958). After incubation less than 2% of the activity remained in the eluate, confirming that the fixation product was in fact L-[4-^{14}C]aspartate. It has previously been shown that L-[4-^{14}C]aspartate is

Table 1. *Incorporation of $^{14}CO_2$ into aspartate in reaction mixtures containing* Kalanchoë *enzyme, phosphoenolpyruvate and $^{14}CO_2$ together with the additions listed below*

In this experiment 0·3 ml. of tris buffer (20%, w/v; pH 7·4), 0·9 ml. of $MgCl_2$ (30 μmoles) and 3 ml. of *Kalanchoë* enzyme were added to each of four 8 ml. reaction vessels together with the additions shown below. At zero time 0·5 ml. of a solution containing potassium phosphoenolpyruvate (10 μmoles at pH 7·4) and sodium [^{14}C]bicarbonate (2 μmoles, 20 μc) was injected through the rubber cap of each vessel. After 40 min. the reactions were stopped by adding formic acid (1 ml.) and boiling for 2 min. The volume of each reaction mixture was then adjusted to 10 ml. and samples (0·025 ml.) were chromatographed (see Methods). Volume of each of the additions listed was 0·3 ml.

Additions	10^{-4} × Counts/min. in aspartate	10^{-4} × Counts/min. added*	Percentage of CO_2 fixed
Water	0·44	146	0·3
NH_4Cl (30 μmoles)	0·32	146	0·2
Glutamate (30 μmoles)	121	146	83
Aspartate (30 μmoles)	126	146	86

* 146×10^4 counts/min. = 10 μc under conditions of measurements.

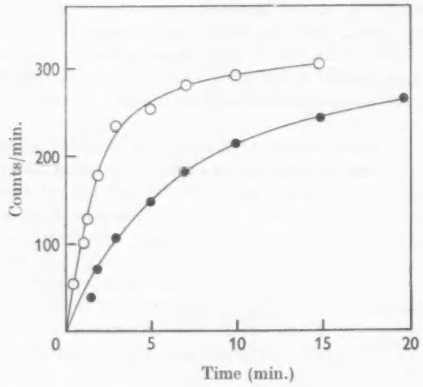

Fig. 1. Rate of incorporation of $^{14}CO_2$ into L-aspartate with aspartate or glutamate as the amino donor. At the start each reaction mixture (4 ml.) contained: *Kalanchoë* enzyme (3 ml.); $MgCl_2$ (30 μmoles) and ○, aspartate (10 μmoles) or ●, glutamate (10 μmoles). Each reaction was started by the addition of 0·5 ml. of a solution containing phosphoenolpyruvate (10 μmoles), $NaH^{14}CO_3$ (10 μc, 1 μmole) and tris buffer (10%, w/v; pH 7·4). Samples (0·5 ml.) were taken at the times indicated, added rapidly to hot formic acid and boiled for 2 min. Radioactivity was counted directly on one-dimensional chromatograms.

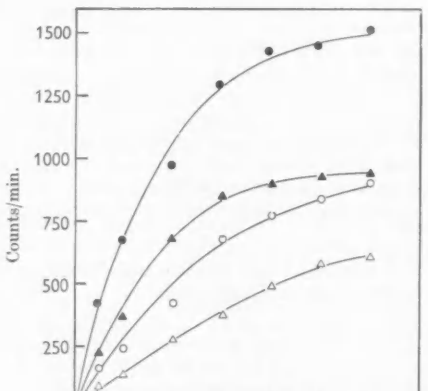

Fig. 2. Rate of incorporation of $^{14}CO_2$ into aspartate with aspartate and glutamate as the amino donor. Reaction mixtures were as described for Fig. 1 but with a different sample of *Kalanchoë* enzyme and ●, aspartate (2 μmoles), ○, glutamate (2 μmoles), ▲, aspartate (1 μmole), △, glutamate (1 μmole).

the major product of the corresponding system in which glutamate is the amino donor (Dearing & Walker, 1960).

Exchange transamination between glutamate and α-oxoglutarate

The exchange between glutamate and its keto acid analogue (reaction 2) has been previously demonstrated with crude (Nisonoff *et al.* 1954) and highly purified (Jenkins *et al.* 1959) preparations from pig heart, but it seemed desirable to repeat these experiments with an enzyme from a plant source. The route by which $^{14}CO_2$ is introduced into α-oxoglutarate and glutamate during the course of feeding experiments is not certainly known, but, at least in the dark, the reactions of the Krebs cycle are probably involved (see e.g. Kunitake, Stitt & Saltman, 1959). Accordingly an extract of mitochondria (see Methods) was used in the following experiment rather than the extract of *Kalanchoë* leaves. The actual site (within the leaf) of the *Kalanchoë* enzymes used in the previous experiment has not been determined but there is evidence that both phosphopyruvate carboxylase and glutamate–aspartate transaminase are present in chloroplasts (Rosenberg, Capindale & Whatley, 1958).

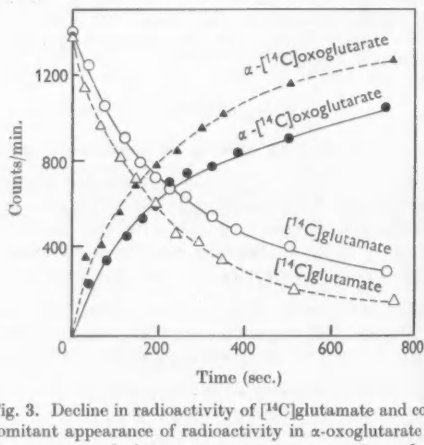

Fig. 3. Decline in radioactivity of [^{14}C]glutamate and concomitant appearance of radioactivity in α-oxoglutarate in the presence of glutamate–aspartate transaminase from *Ricinus* mitochondria. At the start each reaction mixture (1·3 ml.) contained *Ricinus* enzyme (1 ml.), and △, ▲, oxaloacetate (10 μmoles), or ○, ●, α-oxoglutarate (10 μmoles). Each reaction was started by the addition of glutamate (1·6 μc, 0·16 μmole). Samples (0·02 ml.) were taken at the times indicated and pipetted directly on to a spot of formic acid on chromatography sheets and dried with a hot-air blower. Radioactivity in glutamate and α-oxoglutarate was counted directly on the chromatograms after development in butanol–acetic acid (see Methods).

Reaction mixtures were prepared in which [^{14}C]glutamate was incubated with mitochondrial transaminase and either oxaloacetate or α-oxoglutarate. Samples were taken after suitable time intervals and chromatographed as before (Fig. 3).

As the reaction proceeded the level of radioactivity in glutamate fell and there was a concomitant rise in the level of activity in α-oxoglutarate. The initial rate of the exchange transamination (in which α-oxoglutarate was the amino acceptor) was about 1·5 times that of the normal transamination (in which oxaloacetate was the amino acceptor).

DISCUSSION

Partially purified extracts of *Kalanchoë crenata* leaves are known to contain phosphopyruvate carboxylase (Walker, 1957) and glutamate–aspartate transaminase (Walker & Ranson, 1958). In the presence of phosphopyruvate, $^{14}CO_2$ and glutamate such extracts catalyse the synthesis of L-[4-^{14}C]-aspartate (Dearing & Walker, 1960) and it would seem clear that the carboxylation

$$HO_2C{\cdot}CO{\cdot}PO(OH)_2{:}CH_2 + {}^{14}CO_2 + H_2O \rightarrow HO_2C{\cdot}CO{\cdot}CH_2{\cdot}{}^{14}CO_2H + H_3PO_4 \quad (4)$$

and the transamination

$$HO_2C{\cdot}CO{\cdot}CH_2{\cdot}{}^{14}CO_2H + HO_2C{\cdot}CH_2{\cdot}CH_2{\cdot}CH(NH_2){\cdot}CO_2H \rightarrow HO_2C{\cdot}CH(NH_2){\cdot}CH_2{\cdot}{}^{14}CO_2H + HO_2C{\cdot}CH_2{\cdot}CH_2{\cdot}CO{\cdot}CO_2H$$

are linked to bring about the overall reaction:

$$\text{Phosphopyruvate} + {}^{14}CO_2 + \text{glutamate} + \text{water} \rightarrow [4\text{-}{}^{14}\text{C}]\text{aspartate} + \alpha\text{-oxoglutarate} + \text{orthophosphate}. \quad (5)$$

In the present experiments, when aspartate was substituted for glutamate, the only stable labelled product was again L-[4-^{14}C]aspartate. It is known (Nisonoff *et al.* 1954; Jenkins *et al.* 1959) that glutamate–aspartate transaminase from pig heart can catalyse the transfer of an amino group from glutamate to its keto acid analogue (α-oxoglutarate) and we have demonstrated that this reaction occurred in the presence of *Ricinus* mitochondrial extracts. It is not unreasonable that the corresponding transamination between aspartate and its keto acid analogue (reaction 3) should also occur, and in fact our results with the *Kalanchoë* enzyme are consistent with the view that reaction (4) can be linked with reaction (3):

$$HO_2C{\cdot}CO{\cdot}CH_2{\cdot}{}^{14}CO_2H + HO_2C{\cdot}CH(NH_2){\cdot}CH_2{\cdot}CO_2H \rightarrow HO_2C{\cdot}CO{\cdot}CH_2{\cdot}CO_2H + HO_2C{\cdot}CH(NH_2){\cdot}CH_2{\cdot}{}^{14}CO_2H$$

to bring about the overall reaction

$$\text{Phosphopyruvate} + {}^{14}CO_2 + \text{aspartate} + \text{water} \rightarrow \text{oxaloacetate} + [4\text{-}{}^{14}\text{C}]\text{aspartate} + \text{orthophosphate}. \quad (6)$$

According to Jenkins *et al.* (1959) 'exchange transamination' between α-oxoglutarate and glutamate (reaction 2) may proceed at a greater rate than the normal transamination (reaction 1) between α-oxoglutarate and aspartate. This was also our experience with extracts of *Ricinus* mitochondria. Similarly the initial accumulation of label in aspartate in reaction (6) was about three times as rapid as the corresponding accumulation in reaction (5). If glutamate–aspartate transaminase catalyses both types of transamination then the relative rapidity of the exchange reaction may be a consequence of an alignment of the substrates on the enzyme surface such that the transfer of an amino group between carbon chains of equal length (reactions 2 and 3) proceeds more rapidly than the transfer between carbon chains of unequal length (reaction 1).

Our results affect the interpretation of feeding experiments with $^{14}CO_2$. Unless exchange transamination is completely inhibited by the substrates of the normal transamination some exchange transamination will occur whenever glutamate–aspartate transaminase and its substrates come together. In these circumstances $^{14}CO_2$ could appear in aspartate or glutamate either in the absence of synthesis (in the usual sense) or even during metabolic breakdown of these amino acids. It would appear that the rate of the exchange transamination (as compared with the normal transamination) would not be the limiting factor in any accumulation of label by this mechanism.

When crassulacean plants such as *Kalanchoë crenata* are fed with $^{14}CO_2$ in the dark, label appears first in malate and aspartate [for a recent review see Ranson & Thomas (1960)] and subsequently in other compounds. Although there is a striking diurnal fluctuation in the malate content of such plants there does not appear to be any corresponding fluctuation in the level of amino acids (Lyndon, 1957). This anomaly could be explained by the present findings, since label could accumulate in aspartate (and possibly other amino acids) in the absence of net synthesis. Our results would also explain the finding of Rosenberg *et al.* (1958) that fixation of carbon dioxide in the dark in aspartate by chloroplast extracts was increased

'when in addition to phosphoenolpyruvate either alanine, glutamine or aspartic acid was added to the reaction mixture'.

The appearance of $^{14}CO_2$ in certain amino acids during photosynthetic carbon dioxide fixation in a variety of organisms might also be attributed, at least in part, to exchange transamination. Work is at present being undertaken in this laboratory in an attempt to determine the contribution of exchange transamination to the accumulation of label in aspartate by *Chlorella* during photosynthesis and fixation of carbon dioxide in the dark.

SUMMARY

1. In the presence of phosphoenolpyruvate, L-aspartate and an extract of *Kalanchoë crenata*, $^{14}CO_2$ has been introduced into the β-carboxyl group of L-aspartate. The reaction is attributed to an initial carboxylation of phosphoenolpyruvate to yield oxaloacetate followed by an exchange transamination in which aspartate functions as the amino donor.

2. The rate of accumulation of ^{14}C in aspartate has been compared with that of the corresponding reaction, in which the initial carboxylation is followed by a normal transamination in which glutamate serves as the amino donor, and has been shown to be more rapid.

3. The rate of the normal transamination between glutamate and oxaloacetate has been compared with that of the exchange transamination between glutamate and α-oxoglutarate in reactions catalysed by an enzyme from *Ricinus* mitochondria.

4. The results have been discussed in relation to their bearing on $^{14}CO_2$-feeding experiments.

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The Isolation of Bradykinin, a Plasma Kinin from Ox Blood

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Bradykinin belongs to a group of peptides which stimulate smooth muscle and have been given the generic name of plasma kinin; this name has been adopted by general consent of most workers in the field (see Lewis, 1958). The formation of bradykinin from ox-plasma proteins by the action of trypsin or the venom of the snake, *Bothrops jararaca*, was first observed by Rocha e Silva, Beraldo & Rosenfeld (1949). Blood and many other tissues are known to contain enzymes which can be activated under certain conditions and will then cause liberation of a plasma kinin from the plasma proteins (Frey, Kraut & Werle, 1950). Bradykinin is pharmacologically indistinguishable from other plasma kinins (Lewis, 1958; Gaddum & Horton, 1959; Werle, 1953; Holdstock, Mathias & Schachter, 1957; Mathias & Schachter, 1958; Hilton & Lewis, 1956).

The experiments described in this paper have led to the isolation of bradykinin, which is the first member of the plasma-kinin group to be obtained in the pure state.

EXPERIMENTAL

Materials. $(NH_4)_2SO_4$ was a commercial grade. Ammonium acetate was A.R. grade. Ammonium acetate buffer, pH 5, was prepared from stock solutions of 10M-

ammonium acetate and 10M-acetic acid. The solutions were diluted to the required molarity and then the acetic acid solution was added to the ammonium acetate solution until pH 5 was reached (glass electrode). Approximately 160 ml. of acetic acid soln./500 ml. of ammonium acetate soln. was required.

Carboxymethyl(CM)-cellulose was prepared from Solka Floc cellulose powder by a slight modification of the method of Peterson & Sober (1956). Before its conversion into the carboxymethyl derivative, fine particles were removed from the cellulose by sedimentation. The cellulose powder was stirred with about 6 vol. of water and the mixture was allowed to settle for several hours. Particles which had not settled by this time were rejected by decantation. The process was repeated several times. The sludge was filtered, washed with methanol and dried in air. After the reaction of 60 g. of cellulose with chloroacetic acid and addition of 500 ml. of 10% acetic acid to the reaction mixture, as described by Peterson & Sober (1956), a further addition of acetic acid was made, with stirring, until a pH of 5–6 was obtained. Fine particles were removed by sedimentation as before. It was then transferred to a glass tube 7 cm. × 50 cm., fitted with a filtration plate at the bottom and washed with 10% (v/v) acetic acid until the flame test in the eluate was negative. It was washed with 2 l. of water, transferred to a filter funnel, washed with ethanol and dried in air. The yield was approx. 20 g.

Except in two-dimensional chromatography butan-1-ol was redistilled.

Trichloroacetic acid was A.R. grade, redistilled at 65°/15 mm. Hg.

Ether (d 0·720) was allowed to stand for several days over NaOH flakes and redistilled.

Phenol–water for chromatography was prepared by adding 5 vol. of liquid phenol to 2 vol. of water and dissolving in the mixture 0·0003% of ethylenediaminetetra-acetic acid (EDTA).

Butanol–acetic acid for chromatography was prepared by shaking together butan-1-ol, water and acetic acid (63:27:10, by vol.) and keeping for several days before using the upper layer.

Bradykinin at P 4 stage and subsequent stages was handled in glassware which had been treated with a solution of dimethyldichlorosilane in carbon tetrachloride and washed with warm water.

Fractionation of ox blood. Fresh ox blood was collected at the slaughterhouse and defibrinated immediately. The red cells were separated by centrifuging in a De Laval Industrial Separator, type 1229G, at room temperature with a gravity disk of 37 mm. internal diam. The rate of feed was 90 l./hr. and the yield of serum was about 44 l. To this amount of serum was added, with mechanical stirring, 22 l. of $(NH_4)_2SO_4$ soln. saturated at room temperature. The ppt. was removed as completely as possible by centrifuging once in a Sharples no. 5 Centrifuge with a 5 mm. diam. jet and the supernatant (60 l.) was treated with 13 l. of saturated $(NH_4)_2SO_4$. Except that a 2 mm. diam. jet was used, the ppt. was collected by centrifuging as before, dissolved in 5 l. of water and dialysed in Visking dialysis tubing against deionized tap water. The latter was prepared with the aid of a Permutit Portable Deminrolit Plant MK 4. Dialysis was continued until SO_4^{2-} ion had been removed. The final volume was 9 l.

Preparation of crude bradykinin. The substrate solution, prepared as described above, was treated with 90 ml. of 10N-HCl and then heated at 37° for 30 min. with gentle stirring (cf. Gaddum & Horton, 1959). The pH of the solution was adjusted to 7·5 with 4N-NaOH and incubated for 6 hr. at 37° with crystallized salt-free trypsin (Armour Pharmaceutical Co. Ltd., Eastbourne). The trypsin was added in portions of 75, 37·5 and 37·5 mg. respectively at zero time, 2 hr. and 4 hr. from the commencement of the incubation. The solution was then poured rapidly into 30 l. of boiling ethanol and when the mixture had cooled it was centrifuged and the ppt. was discarded. The supernatant was evaporated under reduced pressure at a temperature not exceeding 40° to a volume of approximately 300 ml. Redistilled butanol was added in small portions to this solution, with gentle shaking, until no more would dissolve. Trichloroacetic acid solution (50%, w/v) was then added to bring the concentration of trichloroacetic acid in the solution to 0·3%. The resulting solution formed the first lower phase of a countercurrent-distribution process carried out in five bottles. The solvent system was prepared by equilibration of equal volumes of butanol and aqueous 1% (w/v) trichloroacetic acid solution. The volumes of upper and lower phases were equal to each other and to the volume of the crude bradykinin soln. forming the lower layer in the first bottle. Emulsions which formed were separated by centrifuging. The process was continued until all bottles were filled with both layers, the upper layer forming the moving phase. The upper phase in the fifth bottle was removed and equilibrations were continued, with addition of a fresh upper phase to the first bottle, until all bottles were again filled. The lower phases in the first two bottles were rejected. The upper phase that had been removed from the fifth bottle was mixed with twice its volume of peroxide-free ether and equal volumes of water and the mixture were equilibrated. The resulting upper layer was rejected and the aqueous phase was combined with the remaining upper and lower phases. The two-phase mixture was evaporated under reduced pressure to a volume of about 200 ml. at a temperature not exceeding 35°. The mixture was fed continuously into a cyclic evaporator, it being so arranged that the upper (butanol) layer formed the major portion of the liquid entering the evaporator until it had all been removed. The aqueous solution was treated with 3N-HCl until pH 1 (glass electrode) was reached and was then extracted nine times with equal volumes of peroxide-free ether, and the ether extracts were rejected. After the fifth extraction the pH of the aqueous layer was readjusted to 1. The aqueous solution was freed of ether by heating it for about 10 min. under atmospheric pressure in a steam bath and then heating it for a few minutes under reduced pressure at about 35°. It was then freeze-dried, yielding a pale-yellow powder. The amount obtained varied considerably, but on average was about 5 g. It was stored in a vacuum desiccator at −2° over NaOH pellets. The crude material (P2) was stable for long periods under these conditions.

Biological assay of bradykinin. The biological activity of samples was estimated by assay on the isolated guinea-pig intestine against a standard sample of P2 which had been set aside at the beginning of this investigation. Results were expressed in terms of the weight of P2 to which the test sample was equivalent in biological activity. The terminal portion of the guinea-pig ileum was suspended in 15 ml. of oxygenated Tyrode solution and maintained

at 34°. Contractions of the tissue were recorded by means of a frontal lever writing on a smoked drum of a kymograph.

Chromatography of bradykinin on carboxymethylcellulose. Columns were run at 20°; they were packed without application of external pressure, but during equilibration the head of liquid used generally caused further settlement. Buffer solutions were saturated with toluene to inhibit growth of micro-organisms. Chromatography of P2 was carried out on 32 g. of CM-cellulose in a column of 3·2cm. diam. through which 0·01 M-ammonium acetate had been passed at approx. 50 ml./hr. until the pH values of the influent and effluent fluids were identical. The pH of the solution was not accurately controlled and varied with different batches of the A.R.-grade ammonium acetate used; it was generally pH 6·4–6·5. A portion (4 g.) of P2 was dissolved in 100 ml. of 0·01 M-ammonium acetate and allowed to run into the column. The column was then washed with 0·01 M-ammonium acetate soln. at the rate of 50 ml./hr. until the extinction of the effluent at 280 mμ was less than 0·02. Gradient elution was then commenced with 0·2 M-ammonium acetate in the reservoir and with a mixing vessel of 250 ml. capacity. In this and other gradient experiments the mixing vessel had only two outlets, one to the column and the other to the reservoir; it had no outlet to the atmosphere. The upper reservoir was open to the air. The effluent was collected in fractions of 30 ml. Fractions containing biological activity were combined, boiled for 2 min., cooled and evaporated to dryness under reduced pressure at a temperature of approx. 35°. The evaporation was completed in a rotary evaporator and the syrupy residue heated at 50°/0·01 mm. Hg until ammonium acetate had been removed. This product was described as P4.

Chromatography of P4 was carried out on 2 g. of CM-cellulose in a column of 9 mm. diam. which had been equilibrated with 0·01 M-ammonium acetate buffer, pH 5, in the manner already described. A portion (50 mg.) of P4 was dissolved in 0·1 ml. of 0·01 M-ammonium acetate buffer and, after allowing liquid to drain from the top of the column, the solution was added to the column and allowed to soak in. Two successive portions of 0·1 ml. of the buffer were then added in a similar way. The column was then washed with 0·01 M-ammonium acetate buffer, pH 5, which was also 1 mM with respect to EDTA. The hydrostatic head was adjusted to give a flow rate of 5–6 ml./hr and washing was continued until at least 80 ml. of effluent had been collected. The washing fluid was then changed to 0·01 M-ammonium acetate, pH 5; 40 ml. of effluent was collected. Gradient elution was then commenced with a mixing vessel of 100 ml. capacity and 0·5 M-ammonium acetate in the reservoir. Under these conditions the gradient was approximately linear. Tubes containing biologically active material were placed in a vacuum desiccator over NaOH and H_2SO_4, until a solid residue remained. They were then placed in a wide glass tube closed at one end and open at the other and fitted with a side arm near the open end. The position of the side arm was above the open ends of the tubes containing the solid. The tube was stoppered, evacuated to approx. 0·01 mm. Hg and immersed as far as the side arm in a bath of water at 50°. Heating under these conditions was continued for 1 hr. The combined solid was described as P5.

Chromatography of P5 was carried out on 4 g. of CM-cellulose in a column of 9 mm. diam. It was carried out exactly as for P4 with a mixing vessel of identical capacity.

Electrophoresis of P5. This was carried out on Whatman no. 1 filter paper which had been washed by descending solvent flow with 2 N-acetic acid for 3 days and then with water for 1 day. P5 at a concentration of 10 μg./μl. was applied in the form of a band 2–3 mm. wide and 2 cm. less in length than the total width of the paper. The loading was generally about 100 μg./cm. of paper width; occasionally double this amount was applied with successful results. The buffer used was 2 N-acetic acid and the voltage gradient was 25 v/cm. Electrophoresis was continued for 1 hr. in an apparatus similar to that of Durrum (1950). At the conclusion of the experiment the strip was dried in air at room temperature and a marker strip 0·3 cm. wide was cut lengthwise from the centre of the paper. It was stained with ninhydrin and with Sakaguchi reagent. The section of paper containing the bradykinin was cut out and eluted with water by descending capillary flow, water being allowed to drip from the bottom end of the strip. Elution was generally continued for 16 hr. and the eluate was evaporated to dryness in a vacuum desiccator. The recovery of biological activity was about 70 % of that applied to the paper.

Paper chromatography. Two-dimensional chromatograms were carried out with butanol–acetic acid in the first dimension and phenol–water–NH_3 in the second dimension on Whatman no. 1 paper. For one-dimensional chromatography butanol–acetic acid or pyridine–water (80:20, v/v) was used. Chromatograms were dried at 40°. Quantitative amino acid analysis was carried out by the method of Mandelstam & Rogers (1959), a slight modification being made to permit estimation of proline. The chromatogram was developed in butanol–acetic acid on Whatman no. 3 paper, and, after formation of the spots with ninhydrin, all those except the yellow spot of proline were cut out. The remainder of the sheet was moistened by steaming and heated at 110° for 15 min. The yellow spots due to proline became purple and were then cut out. Reproducible results were obtained in the range 0·01–0·1 μM.

Chromatography of dinitrophenyl amino acids (DNP amino acids) was carried out on Whatman no. 1 paper with the *tert.*-pentanol (2-methylbutan-2-ol) system of Blackburn & Lowther (1951) and 1·5 M-phosphate buffer (Levy, 1954), used either singly or for two-dimensional chromatograms. For quantitative work, sections of paper of known area surrounding the spots were cut out, shredded and allowed to stand with 4·5 ml. of 1 % $NaHCO_3$ soln. at room temperature for 10 min. Extinction of the extract was measured at 350 mμ, except with DNP-proline, where measurement was made at 370 mμ. Blank estimations were made on pieces of paper cut from the same sheet.

Use of fluoro-2:4-dinitrobenzene for end-group determination and dinitrophenylation of amino acid mixtures. To the peptide or mixture of amino acids (containing between 0·1 and 1 μequiv. of free amino groups), dissolved in a mixture of 1 ml. of 2 % (w/v) $NaHCO_3$ and 1 ml. of ethanol, was added 5 μl. of fluoro-2:4-dinitrobenzene. The mixture was shaken by hand for a few minutes, when a homogeneous solution resulted; this was allowed to stand in the dark at room temperature for 3 hr. The solution was then extracted three times with an equal volume of ether. In the determination of end groups the aqueous layer was made

acid (pH 1), evaporated to dryness and hydrolysed with 6N-HCl at 115° for 16 hr. DNP amino acids with the exception of DNP-arginine were extracted from acid solution (pH 1 or less) with ether. If much dinitrophenol was present in the ether extract, this was removed by the vacuum-sublimation technique of Mills (1952). DNP-arginine was extracted with butanol.

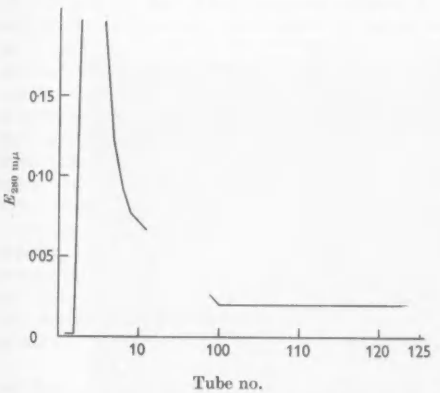


Fig. 1. Results of chromatography of 4 g. of P2 on 32 g. of CM-cellulose at pH 6·4. The fraction volume was 30 ml. The gradient from 0·01 to 0·2M-ammonium acetate was commenced at tube no. 100. Smooth-muscle-stimulating activity was found in tubes nos. 102–125.

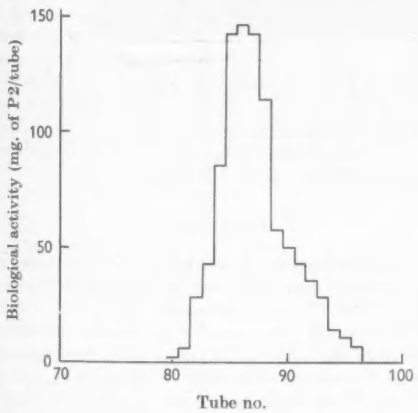


Fig. 2. Results of chromatography of 32 mg. of P4 on 2 g. of CM-cellulose at pH 5. The fraction volume was 1·4 ml. After washing with EDTA (see text) the gradient from 0·01 to 0·5M-ammonium acetate was commenced at tube no. 1. Results are expressed in terms of the wt. of standard P2 to which the tube contents were equivalent in biological activity.

RESULTS

Chromatography of bradykinin on carboxymethylcellulose

Fig. 1 illustrates the results obtained on chromatography of 4 g. of crude bradykinin (P2) on CM-cellulose. It will be seen that a large amount of inactive peptide that absorbs light at 280 mμ emerges in the first 300 ml. of effluent. Further washing with 0·01M-ammonium acetate decreased the extinction of the effluent to about 0·02 at 280 mμ and thereafter it remained almost constant. Tubes nos. 102–125 each contained smooth-muscle-stimulating activity equivalent to more than 5 mg. of P2 and the contents of these were combined. The yield of P4 obtained in this experiment and similar ones was 60–70 mg., which was equivalent in biological activity to 2·4–2·8 g. of P2. The recovery was therefore 60–70%. Inferior results were obtained when chromatography was carried out at 2°.

In Fig. 2 are shown the results of chromatography of P4 on CM-cellulose. The contents of tubes containing biological activity equivalent to more than 10 mg. of P2 were combined. The yield of P5 was 7·8 mg., which was equivalent in biological activity to 925 mg. of P2. An amount of P4 equivalent to 1·4 g. of P2 was used in this experiment and therefore the recovery was 66%.

The results shown in Fig. 3 were obtained on chromatography of 15 mg. of P5 on CM-cellulose. In this experiment there was false indication of incipient separation into two peaks. The contents of tubes nos. 105–110 had the properties of pure bradykinin. The yield was 5·3 mg. It was concluded that the low yield of biological activity in tube 108 was due to a defect in the experimental procedure.

Electrophoresis and paper chromatography of P5

When P5 was submitted to chromatography on Whatman no. 1 paper with butanol–acetic acid as solvent, separation of bradykinin from the inactive peptides was achieved after the chromatogram had been allowed to develop for several days. These results were not sufficiently reproducible to warrant their continued use. At times no separation was achieved and the bradykinin did not leave the origin. It was considered possible that this difficulty was due to contamination with metal ions. Flame-spectrographic analysis (by Johnson, Matthey and Co. Ltd., Wembley, Middx.) revealed that P5 contained 1% by wt. of magnesium. A number of other metallic elements were present, but these were in much smaller quantities than magnesium.

In subsequent experiments paper electrophoresis was used in preference to paper chromatography,

as it was more easily applicable to preparative work. In order to achieve the isolation of pure bradykinin it was necessary to include in the chromatography of P5 a stage of washing with EDTA. Plate 1 shows the results obtained on electrophoresis of P5 prepared with and without the aid of EDTA. It would clearly be impossible to achieve the isolation of pure material from an electrophoresis experiment such as that illustrated in strip no. 1. Strips nos. 2 and 3 show that bradykinin stains weakly with ninhydrin and strongly with the Sakaguchi reagent. By the use of the dipping technique of Jepson & Smith (1953) it was possible to stain the same strip with both ninhydrin and Sakaguchi reagents. The substances stained strongly by ninhydrin on strips nos. 2 and 3 in Plate 1 were biologically inactive.

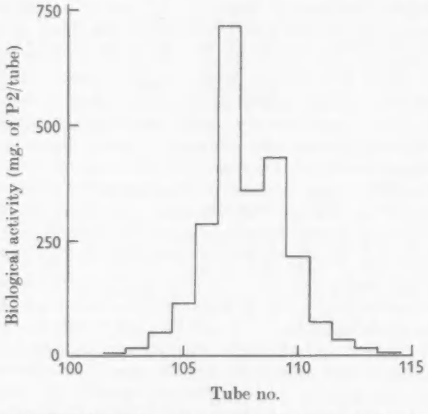


Fig. 3. Results of chromatography of 15 mg. of P5 on 4 g. of CM-cellulose at pH 5. Conditions were identical with those given in Fig. 2.

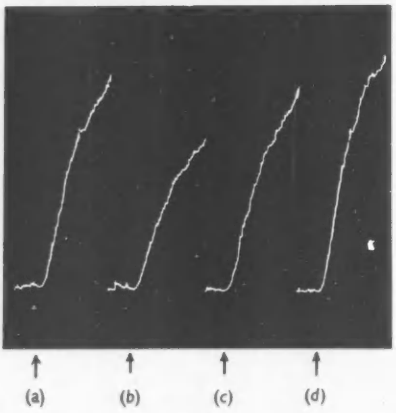


Fig. 4. Responses of the guinea-pig ileum suspended in 15 ml. of Tyrode solution to pure bradykinin (P6), 40 μmg. and 50 μmg. at (*b*) and (*d*) respectively, and to P2, 22·5 μg. and 20 μg. at (*a*) and (*c*) respectively.

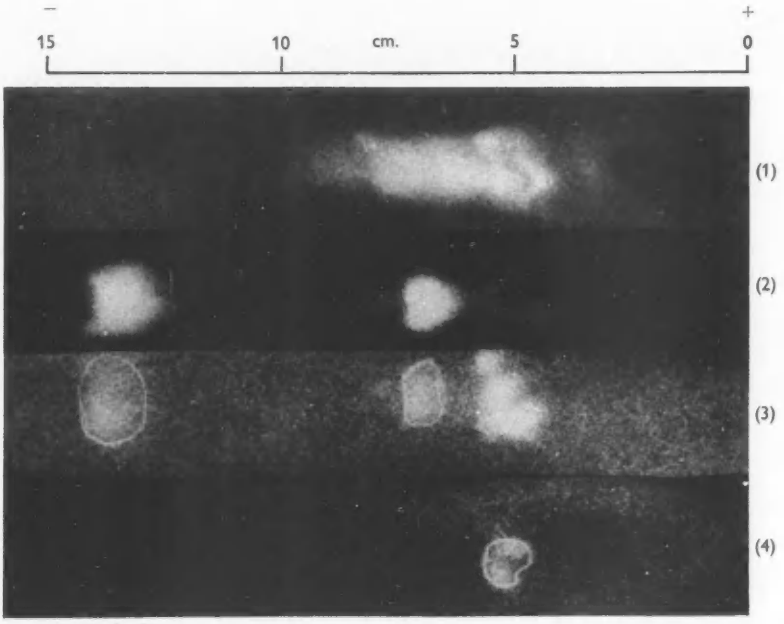
Properties of pure bradykinin

The substance prepared by electrophoresis of P5 as shown in Plate 1 or by further CM-cellulose chromatography of P5 as shown in Fig. 3 was found to contain a single *N*-terminal residue, that of arginine. Hydrolysis followed by two-dimensional paper chromatography revealed the presence of five amino acids, serine, glycine, proline, arginine and phenylalanine. The molar proportions of each amino acid, found by quantitative paper chromatography of the amino acids themselves or of their DNP derivatives, were serine 1, glycine 1, proline 2, arginine 2 and phenylalanine 2. [Degradative work recently carried out by the authors (Elliott, Lewis & Horton, 1960) has revealed that the result obtained for proline is incorrect. The molecule of bradykinin contains three residues of proline.]

Bradykinin was found to possess very high biological potency. For example, the threshold dose for contraction of the isolated guinea-pig ileum was approx. 1 μmg./ml. Fig. 4 shows the results obtained when the biological activity of pure bradykinin (P6) was compared with that of P2. The slow contraction is a characteristic feature of the response of guinea-pig ileum to bradykinin. P6 at a concentration of 3 μmg./ml. produced a marked contraction of the tissue. From these results it was concluded that P6 was 444 times as active as P2. The biological activity of bradykinin was destroyed by chymotrypsin, but not by trypsin. Bradykinin resisted attack by 0·1N-HCl at 100° for at least 1 hr. and by 0·1N-NaOH at room temperature for at least 2 hr.

DISCUSSION

The purification of bradykinin on columns of IRC-50 resin was described by Andrade & Rocha e Silva (1956). These authors used phosphate buffers for elution and were unable to remove inorganic ions from the eluates. In the present investigation it was decided that if a volatile buffer could be used this would be much more convenient than a non-volatile buffer. It was decided to explore the potentialities of CM-cellulose in the chromatography of bradykinin. In the course of another investigation, one of us (E.W.H., unpublished work) found that urinary kinin, a peptide closely related to, if not identical with, bradykinin, was irreversibly adsorbed on to IRC-50 in the presence



Results of four separate electrophoresis experiments performed under identical conditions (see text). (1) Electrophoresis of P5 which had been prepared on a CM-cellulose column without EDTA treatment. The strip was stained with ninhydrin. (2) The same technique applied to P5 from a CM-cellulose column with EDTA treatment. (3) Identical with 2, except that the strip was stained with Sakaguchi reagent after ninhydrin. The spots marked by a (pencil) line were positive only to ninhydrin. (4) Electrophoresis of pure bradykinin. The strip was stained with ninhydrin and Sakaguchi reagents.

of ammonium acetate. Losses of activity occurred in all the CM-cellulose columns used and these could have been due to irreversible adsorption.

It was surprising to find that bradykinin contained magnesium after passage twice through CM-cellulose. Accurate data are not available for the magnesium content of pure bradykinin obtained from EDTA-treated columns, owing to the relatively large amount needed for flame-spectrographic analysis. Qualitative results show that it is still present in the purest material, although in decreased amount compared with P5 prepared without the aid of EDTA.

Recent work has shown (Elliott, Horton & Lewis, 1960) that bradykinin exerts all the actions which have been attributed to one or another of the plasma kinins (smooth-muscle stimulation, vasodilatation, increased capillary permeability, pain production), but the question whether or not the plasma kinins constitute several biologically active peptides or a single peptide (bradykinin) must remain open until other plasma kinins have been isolated and their structures determined.

SUMMARY

1. Bradykinin was formed by trypsin digestion of the fraction of ox-serum proteins which precipitated between 33 and 45% of saturation with ammonium sulphate.

2. Bradykinin was isolated in the pure state by application of the techniques of countercurrent distribution, chromatography on carboxymethylcellulose and paper electrophoresis.

3. Bradykinin contained the five amino acids glycine, serine, proline, phenylalanine and arginine in the molar proportions 1:1:3:2:2 and one of the arginine residues was *N*-terminal.

The authors are indebted to Mr A. W. Hemmings, who devised techniques for the large-scale fractionation of blood, and to Mr R. Blakemore, Miss M. Kinsella, Mr E. Stevens and Mr I. Croker for valuable experimental assistance.

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Preparation of Purified 3-Hydroxyanthranilic Acid Oxidase from Rat and Ox Liver

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Hepatic 3-hydroxyanthranilic acid oxidase is an enzyme which has been studied hitherto without any previous and proper purification, except the only consistent attempt by Stevens & Henderson (1959), who obtained a fourfold purification from an extract of acetone-dried powder of ox liver.

The present work deals with the conditions for obtaining higher purification of the oxidase. The specific activity we have obtained is 50-fold that obtained by Stevens & Henderson (1959). Some properties of the purified enzyme are also reported.

MATERIALS AND METHODS

Chemicals. 3-Hydroxyanthranilic acid (Hoffman–La-Roche, Switzerland), ethanethiol (Schuchardt, Germany), γ-collidine (Merck, Germany); other products were obtained

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as chemically pure compounds from local commercial sources. Ammonium sulphate had been recrystallized from solutions containing 1% of ethylenediaminetetraacetate. Glass-distilled water was used.

Activity test. The 3-hydroxyanthranilic acid-oxidase activity was measured spectrophotometrically by following the increase in extinction at 360 mμ (E_{360}) due to the formation of the primary oxidation product (Bokman & Schweigert, 1951). All measurements were carried out in 0·033 M-collidine buffer prepared according to Gomori (1946) and at room temperature. The temperature is indicated in the graphs; usually it was about 20°.

Definition of activity. The activity unit used by Stevens & Henderson (1959) is the amount giving an initial rate such that the increase in E_{360} is 0·01/min. in 3 ml. of an incubation mixture containing 0·1 μmole of substrate. We preferred, however, to use a different unit based on μm-moles of primary oxidation product formed/min. in the incubation mixture; for this compound we assumed a molar extinction coefficient of $4·7 \times 10^4$ at 360 mμ, as reported by Wiss & Weber (1958). This enzyme unit corresponds to 1·56 Stevens & Henderson (1959) units.

The specific activity is given as units/mg. of protein. Proteins were determined by the biuret method according to Gornall, Bardawill & David (1949).

Apparatus. Spectrophotometric and spectrofluorometric measurements were carried out in a Beckman model DK2 spectrophotometer and in a Farrand double-grating spectrofluorometer respectively.

Extraction of the enzyme. Previous studies on 3-hydroxyanthranilic acid oxidase were carried out either on homogenates (Schweigert & Marquette, 1949; Priest, Bokman & Schweigert, 1951; Rabinowitz, Fineberg & Greenberg, 1953) or on acetone-dried-powder extracts (Bokman & Schweigert, 1950; Long, Hill, Weinstock & Henderson, 1954; Mehler, 1956; Stevens & Henderson, 1959). We observed, however, that it is more convenient to start purification from the supernatant of cell fractions. Rat- and ox-liver homogenates show a specific activity of about 20 whereas the cytoplasmic fraction shows a specific activity of about 100 for rat liver, ox liver being slightly less active. The presence of the 3-hydroxyanthranilic oxidase in the cytoplasmic fraction was also reported by Singal, Sydenstricker & Littlejohn (1953).

Rat-liver homogenates in 0·25 M-sucrose were obtained and fractionated according to Schneider (1948). After the mitochondria had been separated at 24 000 *g* according to Schneider's method, the supernatant was acidified to pH 5·0 by the dropwise addition of 0·1 N-HCl with constant stirring at 0°. The resultant precipitate was centrifuged at 24 000 *g* for 20 min. The supernatant was our 'cytoplasmic fraction'.

Protection of the enzyme. The generally reported instability of 3-hydroxyanthranilic acid oxidase caused the failure of all previous purification attempts (Mehler, 1956; Stevens & Henderson, 1959). Table 1 shows that the removal of oxygen, presence of substrate, addition of Fe^{2+} ions and ethanethiol prevent enzyme inactivation. The activating role of Fe^{2+} ions and reduced glutathione was previously reported by Stevens & Henderson (1959).

Consequently, the purification of 3-hydroxyanthranilic acid oxidase was performed in the presence of 0·1 mM-$FeSO_4$, mM-ethanethiol, 0·2–0·5 mM-3-hydroxyanthranilic acid and in the absence of molecular oxygen.

Purification procedure. Table 2 shows a typical purification procedure in detail. It appears that the precautions described above result in a good yield, the increase in total units being due both to good preservation of the enzyme and the removal of inhibitory substances.

The maximal specific activity was 2700; the purification obtained was 40-fold as compared with the activity of the cytoplasmic fraction.

Heating was performed in a water bath at 55° for 5 min. at pH 6·4, in the absence of oxygen and in the presence of the mentioned protecting substances. Acetone precipitation was carried out at a temperature below 0°, the fraction

Table 1. *Protective effect of various substances on 3-hydroxyanthranilic acid-oxidase activity*

Rat-liver homogenate was kept at 4° for 7 hr., in 0·033 M-collidine buffer, pH 6·5, with or without additions. The 3-hydroxyanthranilic acid-oxidase activity at zero time was 480 units/ml. Reported values are expressed in terms of activity/ml. Temperature of the test, 20°. +, Addition; −, no addition.

N_2	3-Hydroxyanthranilic acid	Ethanethiol	$FeSO_4$	O_2	Units/ml.
−	−	−	−	−	430
+	−	−	−	−	540
−	+	−	−	−	310
−	+	+	−	−	400
−	+	+	+	−	960
+	+	+	+	−	1090
−	−	−	−	+	310

Table 2. *Purification procedure of 3-hydroxyanthranilic acid oxidase from ox liver*

Step	Units/ml.	Volume (ml.)	Total units	Proteins (mg./ml.)	Total proteins (mg.)	Specific activity
Cytoplasmic fraction	480	260	125 000	7·7	2 000	60
After heating at 55° for 5 min. (pH 6·4)	740	250	185 000	5·5	1 380	130
After acetone treatment	2 440	89	218 000	3·6	317	680
After $(NH_4)_2SO_4$ precipitation	10 110	27·5	279 000	4·1	119	2 470

precipitated between 45 and 60% being collected. Dialysis was subsequently performed for 8 hr. at 4° in the absence of oxygen, against 0·033 M-collidine buffer, pH 6·5, containing 0·1 mM-$FeSO_4$ and mM-ethanethiol.

Ammonium sulphate fractionation was performed by collecting the precipitate obtained between 35 and 60% saturation. A second 8 hr. dialysis followed, done under the same conditions as above.

Since this enzyme preparation was sometimes observed to contain traces of reduced haemoglobin, a precipitation procedure with ethanol and chloroform (Tsuchihashi, 1923) was often included after the heating step. The precipitation of haemoglobin was then followed by centrifuging and acetone precipitation.

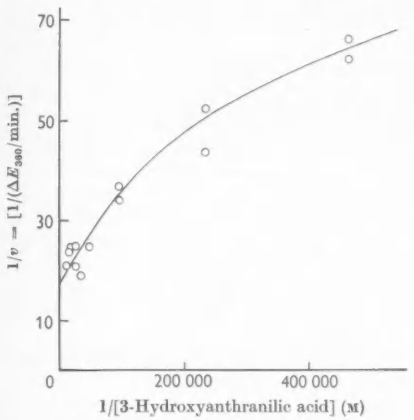

Fig. 1. Relationship between 3-hydroxyanthranilic acid-oxidase activity and 3-hydroxyanthranilic acid concentration. Lineweaver & Burk (1934) reciprocal plots. Temperature 21–22°.

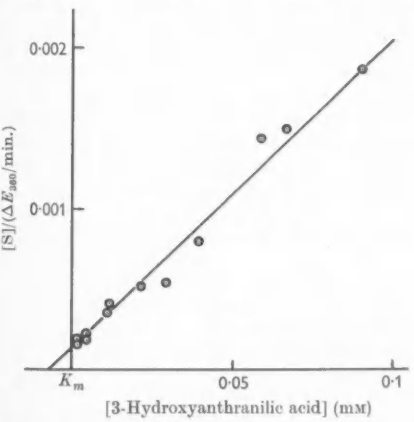

Fig. 2. The experimental values of Fig. 1 in a plot [S]/v versus [S] (according to Haldane & Stern, 1932).

The ethanol–chloroform step generally caused an increase in specific activity, though the final values were not higher than those given in Table 2.

RESULTS

Reaction rate and 3-hydroxyanthranilic acid concentration. By plotting the reciprocal of ΔE_{360}/min. against the reciprocal of the molarity of 3-hydroxyanthranilic acid (Fig. 1) it appears that the relationship between 3-hydroxyanthranilic acid-oxidase activity and substrate concentration cannot be expressed simply in terms of the Michaelis & Menten (1913) equation. Hence, K_m values cannot be calculated from this plot. However, Fig. 2 shows that a plot of [S]/v versus [S] (as according to Haldane & Stern, 1932) gives

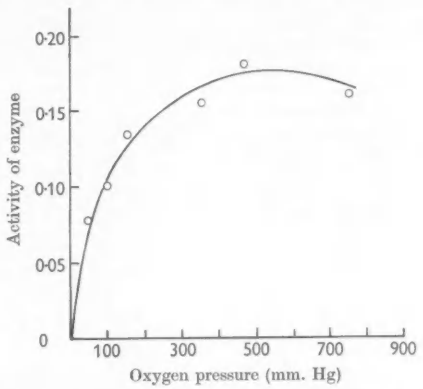

Fig. 3. Relationship between 3-hydroxyanthranilic acid-oxidase activity and oxygen pressure in the incubation mixture. Oxidase activity is expressed as ΔE_{360}/90 sec. Temperature 20–21°.

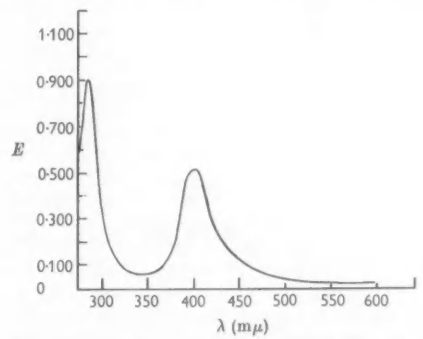

Fig. 4. Absorption spectrum of the purified 3-hydroxyanthranilic acid-oxidase solution (enzyme concentration, 1 mg./ml.).

increased accuracy in a region where the Lineweaver & Burk (1934) plot is least accurate. From Fig. 2 a value of 7 μM can be obtained for K_m.

Reaction rate and oxygen partial pressure. Fig. 3 shows the effects of oxygen partial pressure on the reaction rate measured as ΔE_{360} in 90 sec. Different oxygen pressures were obtained by equilibrating the reaction mixture with different gaseous mixtures before the addition of the enzyme, according to the method used by Boeri & Tosi (1954) in the study of the auto-oxidation of cytochrome *c*. The shape of the curve seems to indicate an inhibition by excess of oxygen.

Spectra of purified enzyme solutions. Fig. 4 shows the spectrum of a solution of purified 3-hydroxyanthranilic acid oxidase. An absorption band at 287 mμ (E 0·910) and another at 400 mμ (E 0·520) are evident. The solution is free from haem groups since it fails to give any haemochromogen spectrum with pyridine and dithionite in alkaline medium. By treatment of the solution with perchloric acid, followed by centrifuging and neutralization, no spectrophotometric evidence of flavins was obtained in the supernatant fluid. No variation of the 400 mμ absorption maximum was observed either in the absence or in the presence of oxygen. Addition of borohydride irreversibly depressed both enzyme activity and absorption maximum at 400 mμ.

Fluorescence spectra. The purified enzyme reveals fluorescence with an activation maximum at 310 mμ and an emission maximum at 415 mμ. The intensity of fluorescence is slightly increased upon addition of borohydride (Fig. 5).

After treatment of the enzyme solution with perchloric acid and then centrifuging and neutralizing, the supernatant fluid shows fluorescence with an activation maximum at 340 mμ and an emission maximum at 500 mμ. Also, in this instance, borohydride caused a slight increase of the emission.

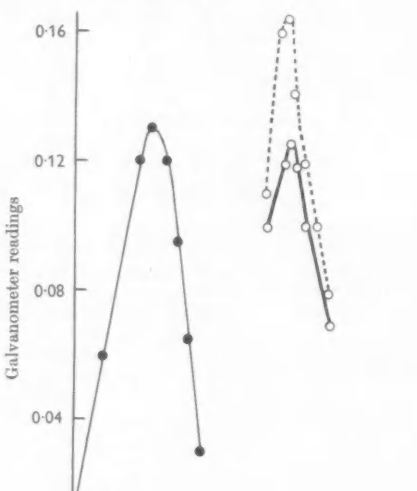

Fig. 5. Fluorescence spectra of the purified 3-hydroxyanthranilic acid-oxidase preparation. Activation spectrum (●—●); fluorescence spectra, with (○- - -○) and without (○—○) borohydride addition. In ●—● the second monochromator was fixed at 420 mμ; in ○- - -○ and ○—○ the first monochromator was fixed at 310 mμ.

DISCUSSION

This work shows the possibility of purifying the oxidase by conventional methods (heating, acetone and ammonium sulphate precipitation). Care must be taken, however, in eliminating molecular oxygen and in operating in the presence of the other substrate, namely 3-hydroxyanthranilic acid, and of Fe^{2+} ions and sulphydryl-group-containing substances. Evidently combination with oxygen impairs activity of the enzyme as, for instance, with cytochrome b_2 (Appleby & Morton, 1958; Boeri & Rippa, 1959).

The purified enzyme functions at a rate which is dependent on the concentration of both substrates (3-hydroxyanthranilic acid and oxygen). K_m for 3-hydroxyanthranilic acid is low (7 μM, when the oxygen pressure is that of air), indicating a great avidity of the enzyme for this substrate.

The dependence of the reaction on the oxygen pressure gives some indication of the role of 3-hydroxyanthranilic acid oxidase in the liver. At the physiological partial pressures of oxygen (20–100 mm. mercury) the enzyme operates at a rate which is far from maximal. The oxidation of 3-hydroxyanthranilic acid by this route is therefore slow and it should be markedly depressed when the oxygen supply is insufficient. The rate of oxidation of 3-hydroxyanthranilic acid in the liver should therefore depend on the integrity not so much of the portal circulation, but rather of the suprahepatic arteries which supply oxygenated blood to the liver.

We do not know as yet whether the 400 mμ absorption could be ascribed to a chromophore on the enzyme molecule or to an impurity. This absorption is not due to haem groups since the enzyme solution fails to exhibit any absorption band between 500 and 600 mμ upon reduction with borohydride, and since there is no evidence of the presence of haemochromogens after pyridine treatment.

The purified preparations seem to be free from flavins, since no spectrophotometric and fluorometric evidence thereof was obtained.

SUMMARY

1. 3-Hydroxyanthranilic acid oxidase has been purified from rat and ox liver. The most active preparations of the enzyme oxidize 2500 μm-moles of 3-hydroxyanthranilic acid/min./mg. of protein.

2. The dependence of the reaction rate on the concentration of both substrates (3-hydroxyanthranilic acid and oxygen) was measured. K_m for 3-hydroxyanthranilic acid is low (7 μM under the conditions of the activity test). At the oxygen pressure of the air the reaction rate is still far from maximal.

3. The purified enzyme solutions present an absorption maximum at 400 mμ and a peculiar fluorescence. There is not yet sufficient evidence to ascertain whether these bands are due to the enzyme itself or to impurities.

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The Metabolism of C_2 Compounds in Micro-organisms

6. SYNTHESIS OF CELL CONSTITUENTS FROM GLYCOLLATE BY *PSEUDOMONAS* SP.*

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The growth of micro-organisms on C_2 compounds as the sole source of carbon necessitates the occurrence of reactions whereby the C_2 substrate provides both metabolic energy and the carbon skeletons of cellular constituents. When the C_2 substrate is acetate, both these functions are fulfilled by the tricarboxylic acid cycle, with the glyoxylate cycle operating in addition to maintain the concentrations of intermediates utilized for growth. But this combination of reactions cannot explain growth on C_2 compounds which are more highly oxidized than acetate, such as glycollate. Moreover, though studies with a mutant of *Escherichia coli* confirmed that the tricarboxylic acid cycle plays an essential role in growth on glycollate, they showed that this C_2 substrate was oxidized via a dicarboxylic acid cycle in the absence of a functional tricarboxylic acid cycle (Kornberg & Sadler, 1960). A different sequence of reactions must therefore occur to replenish from glycollate the supply of intermediates of the dicarboxylic and tricarboxylic acid cycles drained away to provide cell materials for the growing bacteria.

The main purpose of this paper is to present evidence for the nature of such a biosynthetic

* Part 5: Kornberg, Phizackerley & Sadler (1960).

sequence in *Pseudonomas* sp. growing on glycollate as sole carbon source. The results obtained further support the view that, like *E. coli*, *Pseudomonas* sp. derive metabolic energy under these conditions from the oxidation of glycollate via a dicarboxylic acid cycle in which glyoxylate is completely oxidized by a series of reactions in which acetyl-coenzyme A, malate, oxaloacetate and pyruvate play a catalytic role.

An outline of similar work with *Pseudomonas ovalis* Chester has been previously published (Kornberg & Gotto, 1959).

MATERIALS AND METHODS

Maintenance and growth of organisms. The original culture of *Pseudomonas* (B_2aba) was a gift from Dr J. Lascelles. The organisms were maintained on slopes containing 25 mM-sodium glycollate, 25 mM-NH_4Cl, 25 mM-sodium–potassium phosphate, pH 7·2, and salts (2 mg. of $CaCl_2,6H_2O$, 0·2 mg. of $MnSO_4,4H_2O$, 4 mg. of $MgSO_4,7H_2O$ and 0·2 mg. of $FeSO_4,6H_2O$/100 ml. of medium), solidified with 2% (w/v) agar agar (Hopkin and Williams Ltd., Chadwell Heath, Essex). Cultures of the organism were grown at 30° and stored at 2°; they were subcultured at intervals of 2–3 weeks.

For experimental use, cells were grown aerobically with shaking at 30°, in Carrel culture flasks (J. A. Jobling and Co. Ltd., Sunderland) containing 300 ml. of growth medium similar to the above but with the carbon and nitrogen sources at a concentration of 50 mM and the agar agar omitted. The bacteria were harvested by centrifuging at 0°, 1500 *g* for 15 min. during the logarithmic phase of growth (at cell densities of 0·3–0·4 mg. dry wt./ml.). For experiments on the incorporation of isotope from [^{14}C]-glycollate by growing bacteria, the packed cells were resuspended in fresh growth medium containing 10 mM-sodium glycollate. For the preparation of extracts or studies on the oxidation of substrates, the packed cells were washed with 0·9% (w/v) KCl and suspended in 0·9% (w/v) KCl.

Incorporation of isotope from [^{14}C]glycollate. The procedures used for studies on the incorporation of [^{14}C]glycollate into constituents of the ethanol-soluble fractions of bacteria, growing on glycollate, were similar to those described for *Pseudomonas* (Kornberg, 1958) and *E. coli* (Kornberg, Phizackerley & Sadler, 1960) metabolizing [^{14}C]acetate.

Assay and identification of labelled compounds. Labelled materials were located and identified on two-dimensional paper chromatograms by radioautography and co-chromatography with authentic unlabelled materials as described by Kornberg (1958). In addition, glycollic acid and glyceric acid were analysed by one-dimensional chromatography in ethanol–ammonia–water (80:4:16, Long, Quayle & Stedman, 1951), and amino acids by one-dimensional chromatography in water-saturated phenol in the presence of ammonia vapour (Consden, Gordon & Martin, 1944) and in the water–diethylamine–butanol–ethanol (5:2:10:10) system of Hardy, Holland & Nayler (1955). The phosphate groups of phosphorylated compounds were removed by incubation with Polidase-S (Schwarz Laboratories Inc., New York, U.S.A.) as described by Benson, Bassham & Calvin (1951); the resultant dephosphorylated compounds were analysed by two-dimensional chromatography (Kornberg, 1958).

Preparation of cell extracts. Freshly grown *Pseudomonas* (B_2aba) were disintegrated either by crushing in the Hughes press (Hughes, 1951), or by subjecting them to the output of a 600 W Mullard magnetostrictor oscillator, operating at 3·5 A, for 2 min. The suspensions, in 0·9% KCl, of disintegrated bacteria obtained by either technique were centrifuged at 24 000 *g* for 15 min. at 0°; the supernatant solutions thus obtained were used for experimental purposes.

Isotopic materials. All isotopic materials were obtained from The Radiochemical Centre, Amersham, Bucks. Sodium [1-^{14}C]glycollate was dissolved in the minimum quantity of water and purified by paper chromatography on Whatman no. 3 paper in ethanol–ammonia–water (80:4:16; Long *et al.* 1951). Sodium [2-^{14}C]glycollate was prepared from the hydrolysis of [2-^{14}C]bromoacetic acid by a procedure modified from that used by Jayasuriya (1956). To 1 ml. of aqueous [2-^{14}C]bromoacetic acid (containing 12 mg. of material and 200 μc of ^{14}C) was added 1 ml. of 1 M-KOH, and the mixture was kept at 80° for 3 hr. The solution was evaporated under O_2-free N_2 to less than 1 ml., cooled to room temperature, neutralized with 6 N-HCl and streaked on to Whatman no. 3 paper. After chromatography in ethanol–ammonia–water (Long *et al.* 1951) the radioactive band on the air-dried paper was located by radioautography and eluted with water. The solution was adjusted to pH 8·0 with 0·2 M-2-amino-2-hydroxymethylpropane-1:3-diol (tris) buffer and stored at −12°.

Sodium [1-^{14}C]glyoxylate was prepared from the calcium salt by treatment with the Na^+ form of Dowex-50 resin; it was assayed by direct counting of the 2:4-dinitrophenylhydrazone isolated on paper chromatograms (El Hawary & Thompson, 1953). [^{14}C]Glyceric acid was prepared enzymically from sodium [1-^{14}C]glyoxylate. A sonic extract of glycollate-grown *Pseudomonas* (B_2aba) (containing 1·0 mg. of protein) was incubated anaerobically at 30° for 15 min., in a Warburg manometer gassed with O_2-free N_2, with 2·5 μmoles of sodium [1-^{14}C]glyoxylate, (giving $1{\cdot}25 \times 10^5$ counts/min. under the conditions of radioassay used), 100 μmoles of potassium phosphate (pH 7·2), 0·5 μmole of thiamine pyrophosphate, 2·5 μmoles of reduced diphosphopyridine nucleotide (DPNH), 10 μmoles of $MgCl_2$ and water to 1 ml. Evolved CO_2 was trapped with 400 μmoles of KOH placed in the centre well. The cup was detached and the contents of the main compartment were mixed with 3 ml. of absolute ethanol. The precipitated material was removed by centrifuging and washed with 1 ml. of 20% (v/v) aqueous ethanol. The combined supernatant solutions were evaporated to a small volume at 60° under O_2-free N_2 and chromatographed two-dimensionally as described by Kornberg (1958). A small portion of the radioactive material was mixed with authentic D(−)-glyceric acid and chromatographed one-dimensionally (Long *et al.* 1951) and two-dimensionally (Kornberg, 1958): the radioactive spot located by radioautography occupied the same position and had the same shape as the carrier D(−)-glyceric acid, which was located by spraying the chromatogram with 0·04% (w/v) ethanolic bromocresol green adjusted to pH 8 with NaOH. The [^{14}C]glyceric acid thus prepared and identified, which contained exactly half

the total radioactivity of the sodium [1-^{14}C]glyoxylate used as starting material, was eluted from the chromatogram with water, adjusted to pH 7 with 20 mM-potassium phosphate, and used for experimental purposes.

Other materials used. Phenazine methosulphate, sodium glyoxylate, coenzyme A and pyridine nucleotides were purchased from Sigma Chemical Co., St Louis, Mo., U.S.A.; 2:6-dichlorophenol-indophenol was from British Drug Houses Ltd., Poole. Generous gifts of lithium hydroxypyruvate were received from Professor F. Dickens, F.R.S.; calcium D(−)-glycerate from Dr P. W. Kent; highly purified sodium glyoxylate from D. I. Zelitch (Connecticut Agricultural Experiment Station, New Haven, U.S.A.); sodium 3-phosphoglycerate from Dr J. R. Quayle; phosphotransacetylase from Mr N. Carr, and sodium pyruvate and oxaloacetic acid from Professor Sir Hans Krebs, F.R.S. All other reagents used were of the highest purity commercially available.

Enzyme assays

Glycollic oxidase. This enzyme (Tolbert, Claggett & Burris, 1949; Zelitch & Ochoa, 1953) was assayed by measuring the reduction of potassium ferricyanide or 2:6-dichlorophenol-indophenol (Zelitch & Ochoa, 1953) or the formation of glyoxylate semicarbazone (Olson, 1959) in a Cary recording spectrophotometer.

In manometric assays of this enzyme, the main compartments of Warburg manometer cups contained 150 μmoles of potassium phosphate buffer, pH 7·2, 0·5 ml. of sonic extract (containing 8–15 mg. of protein) and water to a final volume of 1·7 ml. Strips of Whatman no. 40 filter paper and 400 μmoles of KOH were placed in the centre wells. Sodium glycollate (20 μmoles) and 0·2 ml. of 1% (w/v) phenazine methosulphate were added from the side arm after thermal equilibration at 30°. The gas phase was air. Cups without phenazine methosulphate, without glycollate and without glycollate and phenazine methosulphate were used as controls.

The stoicheiometry of glycollate oxidation was determined by measuring O_2 uptake in the presence of semicarbazide. The procedure was the same as that just described, except that the main compartment of the Warburg flasks also contained 50 μmoles of semicarbazide adjusted to pH 5. At the end of the experiment 0·20 ml. of 12N-HCl was added to the contents of the flasks, and, after centrifuging, glyoxylate was estimated in the supernatant solution by the procedure of Friedemann & Haugen (1943). The 2:4-dinitrophenylhydrazone of glyoxylate was identified by chromatography in the solvent of El Hawary & Thompson (1953).

Glyoxylate carboligase. This enzyme (Krakow & Barkulis, 1956; Krakow, Hayashi & Barkulis, 1959) was assayed manometrically by measuring the rate of anaerobic evolution of CO_2 from glyoxylate. Double-armed Warburg flasks contained 40 μmoles of phosphate buffer, pH 6·4, 0·5 μmole of thiamine pyrophosphate, 10 μmoles of $MgCl_2$, sonic extract of *Pseudomonas* (B_2aba) and water to 1·6 ml. in the main compartments. The flasks were gassed with O_2-free N_2. After thermal equilibration at 30°, sodium glyoxylate (10–30 μmoles) was added from side arm 1. The reactions were terminated by the addition of 0·2 ml. of 50% (w/v) trichloroacetic acid from side arm 2.

When [1-^{14}C]glyoxylate was used as substrate, the centre wells also contained 400 μmoles of KOH but no paper strips. Samples of $Ba^{14}CO_3$ were prepared in duplicate from the contents of the centre wells as described by Sakami (1955) and were counted with a lead-shielded Geiger–Müller mica end-window counter tube. The observed counts/min. were corrected for background radiation and for self-absorption to infinite thinness.

Conversion of glyoxylate into glycerate. The procedure used for measuring the formation of [^{14}C]glycerate from sodium [1-^{14}C]glyoxylate was as described above. When unlabelled glyoxylate was used as substrate, the glycerate formed was isolated by chromatography on Dowex-1 (Cl^- form, ×10, 200–400 mesh) and was estimated by the colorimetric procedure of Bartlett (1959).

Conversion of glycerate into pyruvate. The formation of pyruvate from glycerate was measured by incubating extracts of glycollate-grown *Pseudomonas* (B_2aba) prepared in a Hughes press (Hughes, 1951) with 5 μmoles of sodium D(−)-glycerate, 5 μmoles of glutathione, 10 μmoles of $MgCl_2$, 100 μmoles of phosphate buffer, pH 7·2, 0·20 μmole of adenosine triphosphate (ATP) and water to 1 ml The reaction mixture was incubated at 30° for 30 min. The reaction was terminated by the addition of 0·2 ml. of 50% (w/v) trichloroacetic acid. After centrifuging, pyruvate was estimated as its 2:4-dinitrophenylhydrazone (Friedemann & Haugen, 1943) and identified by chromatography (El Hawary & Thompson, 1953).

The rate of formation of pyruvate from glycerate was determined spectrophotometrically by measuring the rate of decrease in extinction at 340 mμ consequent upon conversion of the pyruvate into lactate in the presence of DPNH and lactic dehydrogenase. Quartz cuvettes (vol. 3 ml., 1 cm. light path) contained 100 μmoles of tris buffer, pH 7·4, 10 μmoles of $MgCl_2$, 5 μmoles of reduced glutathione, 0·2 μmole of ATP, 5 μmoles of sodium D(−)-glycerate, 0·4 μmole of DPNH, 2000 units of crystalline lactic dehydrogenase (C. F. Boehringer und Söhne, Mannheim, Germany) and extracts, prepared in a Hughes press, of glycollate-grown *Pseudomonas*. A cuvette without DPNH was used as the blank. Reactions were followed in a Cary recording spectrophotometer.

Pyruvic oxidase. The pyruvic-oxidase activity of sonic extracts of *Pseudomonas* was measured by the dismutation assay described by Korkes (1955). The evolution of CO_2 was measured with Warburg manometers as pyruvate was dismuted to lactate and acetyl phosphate in the presence of lactic dehydrogenase, phosphotransacetylase, catalytic amounts of diphosphopyridine nucleotide and coenzyme A, phosphate buffer and a sonic extract from glycollate-grown *Pseudomonas* sp. At the end of the reaction acetyl phosphate was estimated as the hydroxamic acid (Lipmann & Tuttle, 1945).

Malate synthetase (Wong & Ajl, 1956), *citrate-condensing enzyme* (Ochoa, Stern & Schneider, 1951) *and* isocitratase (Olson, 1954, 1959; Smith & Gunsalus, 1954, 1955, 1957). These enzymes were estimated spectrophotometrically as described by Dixon & Kornberg (1959).

Malic dehydrogenase. This enzyme was estimated spectrophotometrically by measurements of the rate of decrease in extinction at 340 mμ consequent upon the oxidation of DPNH by oxaloacetic acid. The assay system used was that described previously (Kornberg, 1958).

Pyruvate formation from oxaloacetate. This was estimated spectrophotometrically by measuring the difference in the rates of decrease in extinction at 340 mμ when extracts of

Pseudomonas sp. were incubated with oxaloacetic acid and DPNH in the presence and absence of lactic dehydrogenase. All spectrophotometric assays were performed at $22 \pm 1°$.

RESULTS

Oxidation of substrates by whole cells

Washed suspensions of glycollate-grown *Pseudomonas* rapidly oxidized glycollate, glyoxylate, glycerate, pyruvate and malate, but only slowly oxidized *iso*citrate, α-oxoglutarate and succinate (Table 1). Citrate was not oxidized. The rate of oxygen uptake with glycollate was sufficient to account for the oxidation of 2·1 μmoles of this substrate/mg. dry wt./hr. The total quantities of oxygen absorbed, corrected for endogenous respiration, were 73% of the amounts required for complete oxidation of glycollate or glyoxylate.

Of the substrates tested, malate and succinate were rapidly, and α-oxoglutarate was slowly, oxidized by washed suspensions of the succinate-grown *Pseudomonas* (Table 1). Glycollate was slowly oxidized after a lag period of 30 min., during which time a glycollate 'permease' (for review, see Cohen & Monod, 1957) may have been formed; glycollic oxidase was found in only trace amounts in cell-free extracts of succinate-grown cells.

Incorporation of labelled glycollate

When [1-^{14}C]glycollate or [2-^{14}C]glycollate was added to suspensions of *Pseudomonas* growing on glycollate as sole carbon source, radioactivity was incorporated into both the ethanol-soluble and protein fractions. The incorporation of radioactivity into the ethanol-soluble fraction increased linearly with time for the duration of the experiment (2–3 min.).

Distribution of radioactivity from [2-^{14}C]*glycollate.* The distribution of isotope amongst the labelled components of the ethanol-soluble fractions was analysed by two-dimensional chromatography and radioautography. Label from [2-^{14}C]glycollate rapidly appeared in malate, phosphoglycerate, glycine, citrate and glutamate (Fig. 1). In the samples taken at the earliest times, malate contained the greatest proportion of radioactivity (approx. 28% of the total ^{14}C incorporated). Approx. 25% of the radioactivity was present in phosphoglycerate and 15% in glutamate. Citrate and glycine each contained 13–14% of the total radioactivity, and approx. 4% was found in aspartate. These proportions changed with time: the proportion of total isotope incorporated into glutamate increased and, after 2 min., represented more than 40% of the total radioactivity of the samples. At this time the phosphoglycerate area contained approx. 13% of the incorporated ^{14}C, glycine approx. 6, malate approx. 17, citrate 10 and aspartate 8%. The three curves with the most distinct negative slopes were those of phosphoglycerate, glycine and malate. Initially these three compounds contained approx. 65% of the total

Table 1. *Oxidation of substrates by washed suspensions of* Pseudomonas (B_2aba)

The main compartments of Warburg manometer flasks contained, in 2 ml., 150 μmoles of potassium phosphate, pH 7·2, and washed cells (12 mg. dry wt.). The centre wells contained 400 μmoles of KOH. Substrates as indicated (30 μmoles) were added from the side arms after thermal equilibration at 30°.

Substrate added	Rate of O_2 uptake (μl. absorbed/hr./mg. dry wt. of cells) by cells grown on: Glycollate	Succinate
None	8	10
Glycollate	80	18
Glyoxylate	91	18
Glycerate	50	15
Pyruvate	79	19
L-Malate	52	48
Citrate	8	13
*iso*Citrate	12	10
α-Oxoglutarate	14	24
Succinate	14	71

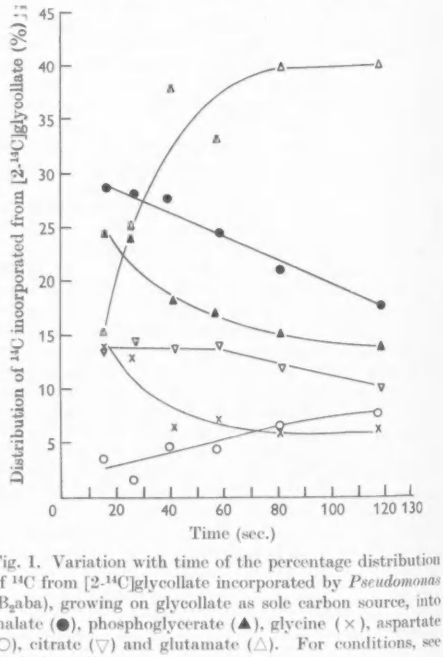


Fig. 1. Variation with time of the percentage distribution of ^{14}C from [2-^{14}C]glycollate incorporated by *Pseudomonas* (B_2aba), growing on glycollate as sole carbon source, into malate (●), phosphoglycerate (▲), glycine (×), aspartate (○), citrate (▽) and glutamate (△). For conditions, see Methods section.

radioactivity, but after 2 min. only 35% of the total radioactivity was found in them. Hence it must be concluded that glycollate enters the metabolic pathways operating in *Pseudomonas* (B_2aba) at sites close to glyoxylate (or glycine), phosphoglycerate and malate.

Oxidation of glycollate by cell extracts

Extracts, obtained by ultrasonic disintegration of glycollate-grown *Pseudomonas* slowly formed glyoxylate from glycollate. The formation of this keto acid, measured spectrophotometrically as the increase in extinction at 252 mμ consequent upon the formation of glyoxylate semicarbazone (Olson, 1959), was dependent upon the presence of cell extract, glycollate and semicarbazide (Fig. 2). The addition of electron acceptors, such as 2:6-dichlorophenol-indophenol, potassium ferricyanide or phenazine methosulphate, greatly stimulated glyoxylate formation and enabled the reaction to be followed manometrically by the uptake of oxygen. Fig. 3 shows results obtained with phenazine methosulphate, which was the most effective of the electron acceptors tested. Although the extract, in the absence of added electron acceptors, catalysed the uptake of 58 μl. of oxygen in 15 min., addition of phenazine methosulphate increased this gas absorption to 205 μl. When this experiment was performed in the presence of semicarbazide, decomposition of the semicarbazone formed with acid 2:4-dinitrophenylhydrazine, and chromatography of the resultant 2:4-dinitrophenylhydrazones (El Hawary & Thompson, 1953) showed glyoxylate to be the sole keto compound present. Comparison of the quantities of oxygen absorbed and the quantities of keto acid produced (Friedemann & Haugen, 1943) established the stoicheiometry of the oxidation of glycollate (Table 2) to be:

$$\text{glycollate} + \tfrac{1}{2}O_2 \rightarrow \text{glyoxylate} + H_2O. \qquad \text{(i)}$$

The marked stimulation of oxygen uptake by phenazine methosulphate, and the yellow colour of

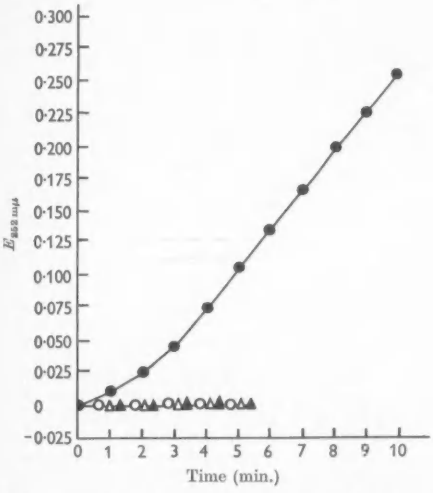


Fig. 2. Formation of glyoxylate from glycollate by sonic extracts of glycollate-grown *Pseudomonas* (B_2aba). The complete system (●) contained in 3 ml., 100 μmoles of potassium phosphate, pH 6·0, 10 μmoles of $MgCl_2$, 60 μmoles of freshly neutralized semicarbazide, 0·06 ml. of a sonic extract of glycollate-grown *Pseudomonas* (B_2aba) (containing 1·0 mg. of soluble protein) and 10 μmoles of sodium glycollate. Glyoxylate formation was measured in a Cary recording spectrophotometer as the rate of change of extinction at 252 mμ consequent upon the formation of glyoxylate semicarbazone (Olson, 1959). From control cuvettes, the extract (○) or glycollate (▲) or semicarbazide (△) was omitted.

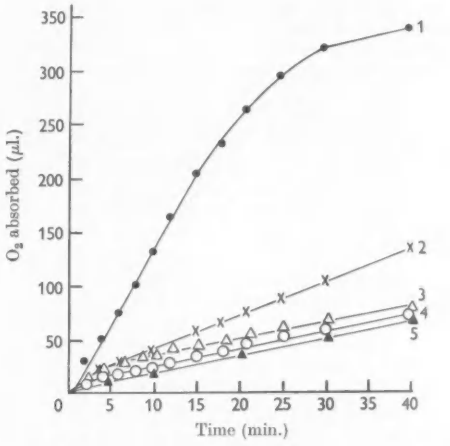


Fig. 3. Oxidation of glycollate by sonic extracts of *Pseudomonas* (B_2aba). The main compartments of Warburg manometer flasks contained in 1·9 ml., 100 μmoles of sodium potassium phosphate, pH 7·2, 10 μmoles of $MgCl_2$, 1·0 ml. of sonic extract of *Pseudomonas* (B_2aba) grown on glycollate or succinate (containing 15 mg. of soluble protein). The centre wells contained 0·2 ml. of 2 M-KOH. After thermal equilibration at 30° for 10 min., 20 μmoles of sodium glycollate and 0·2 ml. of 1% (w/v) phenazine methosulphate were added from the side arms. The quantities (μl.) of O_2 absorbed after tipping the side arms were recorded over 40 min. 1, ●, Complete system, glycollate-grown cell extract; 2, ×, as 1 but phenazine methosulphate omitted; 3, △, as 1 but glycollate omitted; 4, ○, as 1 but glycollate and phenazine methosulphate omitted; 5, ▲, as 1 but containing extract of cells grown on succinate instead of glycollate.

Table 2. *Oxidation of glycollate to glyoxylate by extracts of glycollate-grown* Pseudomonas (B_2aba)

The main compartments of Warburg manometer flasks contained, in 2 ml., 200 μmoles of potassium phosphate, pH 6·0, 10 μmoles of $MgCl_2$, 1·0 ml. of sonic extract of glycollate-grown *Pseudomonas* (B_2aba) (containing 10 mg. of protein) and 60 μmoles of semicarbazide. The centre wells contained 400 μmoles of KOH. After thermal equilibration at 30°, 20 μmoles of sodium glycollate and 0·2 ml. of 1% (w/v) phenazine methosulphate were added from the side arm. At the end of the incubation period the cups were detached, and the contents were acidified with 0·5 ml. of 6N-HCl and centrifuged. The glyoxylate content of the supernatant solution was estimated by the method of Friedemann & Haugen (1943); chromatography of the 2:4-dinitrophenylhydrazones (El Hawary & Thompson, 1953) showed glyoxylate to be the sole keto compound formed.

Contents of side arms	Quantity of O_2 absorbed (μmoles)		Quantity of glyoxylate formed (μmoles)
	Total	Net (corr. for endogenous)	
Complete system	6·2	5·6	11·2
Phenazine methosulphate omitted	3·2	2·6	5·3
Glycollate omitted	0·6	0	0
Complete system, but cell extract boiled before incubation	0	0	0

Table 3. *Stoicheiometry of the anaerobic decarboxylation of glyoxylate*

The main compartment of Warburg manometer cups contained, in 2 ml., 80 μmoles of potassium phosphate, pH 6·4, 0·5 μmole of thiamine pyrophosphate, 10 μmoles of $MgCl_2$ and 1·0 ml. of sonic extract of glycollate-grown *Pseudomonas* (B_2aba) (containing 9 mg. of protein). The manometers were gassed with O_2-free N_2. After thermal equilibration at 30°, sodium glyoxylate contained in the side arms was tipped into the main compartments. After the evolution of CO_2 had ceased, analysis of the contents of the main compartments showed that all the glyoxylate added had been utilized but that a material giving an intense purple colour in the assay of Friedemann & Haugen (1943) had been formed.

Quantity of glyoxylate added (μmoles)	Quantity of CO_2 evolved (μmoles)	Glyoxylate utilized / CO_2 evolved
11	5·5	2·0
22	11·1	1·98
33	16·6	1·98
11*	0	0

* Cell extract boiled before incubation.

Table 4. *Anaerobic decarboxylation of* [1-^{14}C]*glyoxylate*

The complete system contained, in the main compartments of double-armed Warburg manometer cups, 40 μmoles of potassium phosphate, pH 6·4, 0·5 μmole of thiamine pyrophosphate, 10 μmoles of $MgCl_2$, 0·10 ml. of sonic extract of glycollate-grown *Pseudomonas* (B_2aba) (containing 1·0 mg. of protein) and water to 2·0 ml. The centre wells contained 200 μmoles of KOH. The first side arm contained 4 μmoles of sodium [1-^{14}C]glyoxylate (giving $1{\cdot}54 \times 10^5$ counts/min. when assayed as $BaCO_3$ at infinite thinness). The second side arm contained 0·2 ml. of 3N-HCl. The manometers were gassed with O_2-free N_2 and equilibrated at 30°. The reaction was started by tipping the contents of the first side arm into the main compartment; after 30 min., it was terminated by adding the contents of the second. The cups were detached and the contents of the centre wells were mixed with 400 μmoles of Na_2CO_3. The carbon contained in measured portions of this was precipitated as $BaCO_3$ and assayed for radioactivity, the observed counts/min. being corrected for background radiation and self-absorption to infinite thinness. The contents of the main compartments were centrifuged, mixed with 2 μmoles of sodium glyoxylate and chromatographed as the 2:4-dinitrophenylhydrazones (El Hawary & Thompson, 1953).

Contents of cup	10^{-4} × Radioactivity (counts/min.) in			[^{14}C]Glyoxylate utilized / $^{14}CO_2$ evolved
	Glyoxylate (initial)	Glyoxylate (final)	CO_2	
Complete system	15·4	0·0	7·9	1·95
Complete system, but cell extract boiled before incubation	15·4	15·4	0	0

the purified glycollate-oxidizing enzyme, suggest that it is a flavoprotein similar to the glycollic oxidase of spinach (Zelitch & Ochoa, 1953; Frigerio & Harbury, 1958).

Extracts of succinate-grown *Pseudomonas* catalysed the oxidation of glycollate at less than 4% of the rate observed with similar extracts prepared from glycollate-grown cells.

Conversion of glyoxylate into a C_3 compound

When extracts of glycollate-grown cells oxidized glycollate in the absence of semicarbazide as trapping agent, a keto compound accumulated which differed from glyoxylate and formed an orange, insoluble (probably bis) derivative with 2:4-dinitrophenylhydrazine. Glyoxylate was not detected under these conditions, which indicates that the keto compound formed was a product of the further metabolism of glyoxylate.

One such reaction of glyoxylate is its condensative decarboxylation. This reaction was discovered by Krakow & Barkulis (1956) in extracts of glycollate-grown *E. coli*, and the enzyme which catalysed it was named glyoxylate carboligase (Krakow *et al.* 1959). Extracts of glycollate-grown *Pseudomonas* (B_2aba) contained high activities of glyoxylate carboligase. Anaerobically, one molecule of carbon dioxide was evolved for each two molecules of glyoxylate present (Tables 3 and 4). After dialysis of the extracts against pyrophosphate buffer, pH 8·5, the rapid decarboxylation of glyoxylate required the addition of magnesium ions and thiamine pyrophosphate (Fig. 4). These findings are similar to those reported for *E. coli*, where the anaerobic condensation of glyoxylate yielded one molecule of carbon dioxide and one molecule of a C_3 compound, which was assumed to be tartronic semialdehyde or its isomer, hydroxypyruvate [see (ii)].

$$\underset{\substack{\text{glyoxylic}\\\text{acid}}}{2CHO\cdot CO_2H} \xrightarrow[\substack{\text{thiamine}\\\text{pyrophosphate}}]{Mg^{++}} CO_2 + \underset{\substack{\text{tartronic}\\\text{semialdehyde}}}{CHO\cdot CH(OH)\cdot CO_2H} \rightleftharpoons \underset{\substack{\text{hydroxypyruvic}\\\text{acid}}}{CH_2(OH)\cdot CO\cdot CO_2H} \qquad \text{(ii)}$$

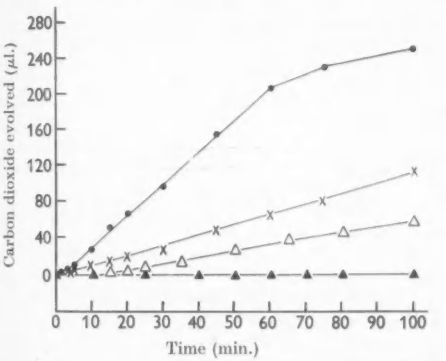


Fig. 4. Evolution of CO_2 from glyoxylate catalysed by extracts of glycollate-grown *Pseudomonas* (B_2aba). The main compartments of Warburg-manometer flasks contained in 1·9 ml., 40 μmoles of potassium phosphate, pH 6·4, 10 μmoles of $MgCl_2$, 0·5 μmole of thiamine pyrophosphate and 0·5 ml. of a sonic extract of glycollate-grown *Pseudomonas* (B_2aba), which had been dialysed against pyrophosphate buffer, pH 8·5, and which contained 2·0 mg. of protein (●). The manometers were gassed with N_2 and equilibrated for 10 min. at 30°. Sodium glyoxylate (30 μmoles) was added from the side arms and the quantities of CO_2 evolved (μl.) were measured. Flasks from which $MgCl_2$ (×), thiamine pyrophosphate (△) or both $MgCl_2$ and thiamine pyrophosphate (▲) had been omitted were used as controls.

Although both isomers of the C_3 product were readily interconverted by extracts of *E. coli* (Krakow & Barkulis, 1956; Krakow *et al.* 1959), extracts of glycollate-grown *Pseudomonas* under these conditions did not form hydroxypyruvate. The conclusion that the C_3 product formed by *Pseudomonas* (B_2aba) extracts differed from hydroxypyruvate rests on the following evidence:

(*a*) When dilute solutions of hydroxypyruvate and the enzymically formed material reacted with acid 2:4-dinitrophenylhydrazine, the reaction product of the latter compound but not of the former rapidly separated out from solution as orange needles, which indicated that a bis derivative had been formed.

(*b*) Alkaline solutions of the 2:4-dinitrophenylhydrazone of hydroxypyruvate are red in colour, and show an absorption maximum at 445 mμ. Similar solutions of the derivative obtained from the enzymically formed C_3 product are deep purple in colour, do not show an absorption maximum at 445 mμ but absorb strongly at 560–570 mμ. The observed spectrum also differs from that noted with solutions of the 2:4-dinitrophenylhydrazones of pyruvate, glyoxylate or glycolaldehyde, but is similar to that obtained with glyoxal or mesoxalic semialdehyde.

(*c*) The 2:4-dinitrophenylhydrazone of hydroxypyruvate formed a compact double spot, close to the lower of the two spots of glyoxylate 2:4-dinitrophenylhydrazone, in the chromatographic solvent system of El Hawary & Thompson (1953). It could be readily separated from the derivative obtained from the enzymic C_3 product, which tended to streak, but the bulk of which ran faster than hydroxypyruvate.

Table 5. *Conversion of* [1-^{14}C]*glyoxylate into* [^{14}C]*glycerate*

The complete reaction mixture contained 100 μmoles of tris buffer, pH 7·4, 0·5 μmole of thiamine pyrophosphate, 10 μmoles of $MgCl_2$, 0·05 ml. of sonic extract of glycollate-grown *Pseudomonas* (B_2aba) (containing 1·0 mg. of protein), 0·45 μmole of DPNH, 0·45 μmole of sodium [1-^{14}C]glyoxylate (giving $2·5 \times 10^4$ counts/min. under the conditions of radioassay used), and water to 3·1 ml. The rate of the reaction was measured as the decrease in extinction at 340 mμ in a Cary recording spectrophotometer. At the end of the incubation, one half of the contents of the cuvettes was pipetted into 3 ml. of ethanol, centrifuged, and the supernatant solution was analysed by chromatography and radioautography (Kornberg, 1958). A portion of the other half was pipetted into tubes containing 0·2 ml. of saturated 2:4-dinitrophenylhydrazine in 2N-HCl. The derivatives formed were extracted into ethyl acetate and analysed by chromatography (El Hawary & Thompson, 1953) and direct radioassay of the labelled spots.

Reaction mixture	$10^{-3} \times$ Radioactivity (counts/min.) of: Glyoxylate (initial)	Glyoxylate (final)	Glycerate
Complete system	25·0	0·10	12·0
DPNH omitted	25·0	0·20	0·80
Complete system but extract boiled before incubation	25·0	25·0	0·00
Complete system; but hydroxypyruvate (1 μmole) added*	25·0	0·15	12·7
DPNH omitted but hydroxypyruvate (1 μmole) added*	25·0	0·20	0·40

* The 2:4-dinitrophenylhydrazone of the hydroxypyruvate, isolated after the incubation, was virtually devoid of radioactivity.

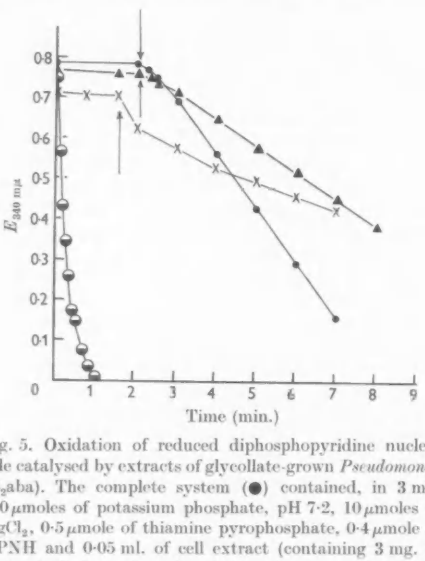


Fig. 5. Oxidation of reduced diphosphopyridine nucleotide catalysed by extracts of glycollate-grown *Pseudomonas* (B_2aba). The complete system (●) contained, in 3 ml., 100 μmoles of potassium phosphate, pH 7·2, 10 μmoles of $MgCl_2$, 0·5 μmole of thiamine pyrophosphate, 0·4 μmole of DPNH and 0·05 ml. of cell extract (containing 3 mg. of protein). Sodium glyoxylate (6·6 μmoles) was added at the point indicated by the arrow. The oxidation of DPNH was measured as the decrease in extinction at 340 mμ in a Cary recording spectrophotometer. Another cuvette (◒) contained the above system but with DPNH omitted; it was incubated for 5 min. at 22° and the reaction was started by addition of DPNH. Control cuvettes contained the complete system but with $MgCl_2$ and thiamine pyrophosphate omitted (▲), or the complete system to which lithium hydroxypyruvate (6·6 μmoles) instead of glyoxylate was added at the point indicated by the arrow (×).

Table 6. *Oxidation of reduced diphosphopyridine nucleotide in the presence of glyoxylate*

The reactions were followed in a Cary recording spectrophotometer at 340 mμ. The 'test' cuvette contained 100 μmoles of potassium phosphate, pH 7·4, 10 μmoles of $MgCl_2$, 0·3 μmole of thiamine pyrophosphate, 0·05 ml. of a sonic extract of glycollate-grown *Pseudomonas* (B_2aba), 0·40 μmole of DPNH and sodium glyoxylate in a total volume of 3·0 ml. In the 'blank' cuvette DPNH was omitted. The product of the reaction was identified as glyceric acid.

Glyoxylate used (μmole)	DPNH oxidized (μmole)	Glyoxylate used / DPNH oxidized
0·10	0·05	2·0
0·30	0·14	2·1
0·50	0·24	2·1

(*d*) The *p*-nitrophenylhydrazone of the enzymic C_3 product was readily separated from the similar derivatives of glyoxal, glycolaldehyde, hydroxypyruvate and glyoxylate in the chromatographic solvents of El Hawary & Thompson (1953).

(*e*) The *p*-nitrophenylhydrazone of the C_3 product formed from [1-^{14}C]glyoxylate was highly radioactive whereas hydroxypyruvate, present throughout the incubation, formed a derivative containing negligible ^{14}C.

(*f*) The enzymic C_3 product reacted approx. 40 times as fast with DPNH and extracts of glycollate grown *Pseudomonas* as did a similar concentration of hydroxypyruvate.

(*g*) The quantities of labelled glycerate formed from labelled glyoxylate in the presence of DPNH and glycollate-grown cell extract (see below) were unaffected by the addition of hydroxypyruvate,

Table 7. *Stoicheiometry of net formation of glycerate from glyoxylate*

The complete reaction mixture contained, in 3 ml., 10 μmoles of potassium phosphate, pH 6·8, 10 μmoles of $MgCl_2$, 0·5 μmole of thiamine pyrophosphate, 5 μmoles of sodium glyoxylate; 5 μmoles of DPNH and 1 ml. of dilute cell extract (containing 0·8 mg. of protein). The system was incubated at room temperature (22°) for 2 hr. After this time, 3 ml. of 95% ethanol were added, the suspensions were centrifuged and the supernatant solutions were passed through Dowex-1 (Cl^- form, ×10, 200–400 mesh). Preliminary tests showed that glyoxal and glycolaldehyde, which might interfere with the colorimetric estimation of glyceric acid, were not retained by the resin. Glyceric acid was eluted from the washed column with 0·05 M-HCl and its concentration determined (Bartlett, 1959) in samples (0·2 ml.) of the eluates evaporated to small known volumes. Glyoxylate was estimated by the procedure of Friedemann & Haugen (1943). The glyoxylate content of tube 2 could not be determined as a material giving a purple colour with 2:4-dinitrophenylhydrazine and alkali (presumably tartronic semialdehyde) was formed.

Tube no.	Contents	Glyoxylate content (μmoles) Initial	Final	Glyoxylate utilized (μmoles)	Glyceric acid content (μmoles) Initial	Final	Glyceric acid formed (μmoles)
1	Complete system	5·0	1·6	3·4	0	1·8	1·8
2	DPNH omitted	5·0	—	—	0	0	0
3	Glyoxylate omitted	0	0	0	0	0	0
4	Complete system, but enzyme boiled	5·0	5·0	0	0	0	0

and the hydroxypyruvate added did not acquire radioactivity during this reaction (Table 5).

These results support the tentative identification of the C_3 product as tartronic semialdehyde.

Conversion of the C_3 compound into glycerate

Cell-free extracts of glycollate-grown *Pseudomonas* oxidized DPNH in the presence of glyoxylate. The reaction started after a short lag period, and required the addition of magnesium ions and thiamine pyrophosphate for maximal activity (Fig. 5). When the extract was preincubated for 5 min. with glyoxylate, the reaction rate was at least 10 times as great as that obtained without preincubation. In the presence of limiting amounts of added glyoxylate, one molecule of DPNH was oxidized per two molecules of glyoxylate added as substrate (Table 6). No accumulation of the C_3 compound was detected when the total amount of glyoxylate added was less than twice the amount of DPNH present. The demonstration of lag period, of the requirements for magnesium ions and thiamine pyrophosphate, of the increased rate after preincubation with glyoxylate and of the stoicheiometry of DPNH oxidation indicated that the process being studied was not a single-step reduction of glyoxylate (possibly to glycollate) but the conversion of glyoxylate into the C_3 compound [reaction (ii)], followed by its reduction to glycerate in the presence of DPNH:

$$\underset{C_3\text{ compound}}{[CHO{\cdot}CH(OH){\cdot}CO_2H]} + DPNH + H^+ \rightarrow \underset{\text{glyceric acid}}{CH_2(OH){\cdot}CH(OH){\cdot}CO_2H} + DPN^+ \quad \text{(iii)}$$

Evidence for the overall conversion of glyoxylate into glycerate [reactions (ii)–(iii)] was obtained by incubating cell-free extracts of glycollate-grown *Pseudomonas* with [1-^{14}C]glyoxylate and DPNH.

Table 8. *Conversion of glycerate into pyruvate by extracts of glycollate-grown* Pseudomonas

In a total volume of 2·0 ml. the complete reaction system contained 100 μmoles of tris buffer, pH 7·4, 10 μmoles of $MgCl_2$, 5 μmoles of glutathione, 10 μmoles of sodium DL-glycerate, 0·5 ml. of extract from glycollate-grown *Pseudomonas* (B_2aba) (containing 3·0 mg. of protein) and varied amounts of ATP. The reaction mixtures were incubated at 30° for 30 min. The reactions were stopped by the addition of 0·2 ml. of conc. HCl. The precipitates were removed by centrifuging. In the supernatant solutions pyruvate was estimated by the method of Friedemann & Haugen (1943), and the identity of the 2:4-dinitrophenylhydrazone was established by chromatography (El Hawary & Thompson, 1953).

Contents	Pyruvate formed (μmoles)
Complete system (0·2 μmole of ATP)	1·2
Complete system (1·0 μmole of ATP)	1·3
Complete system (10 μmoles of ATP)	1·2
Complete system but ATP omitted	0·10
Glycerate omitted	0
Complete system, enzyme boiled	0

For each two molecules of [1-^{14}C]glyoxylate utilized, one molecule of radioactive glycerate accumulated (Table 5); no labelled glycollate was detected. The formation of radioactive glycerate was dependent upon the addition of DPNH. Similarly, the net formation of one molecule of glycerate from two molecules of glyoxylate (Table 7) was shown to be dependent on the presence of glyoxylate, DPNH and cell extract. These results further show that, as expected for reactions (ii) and (iii), the carbon dioxide evolved for the condensative decarboxyl-

ation of glyoxylate arose from the carboxyl groups of one of the glyoxylate molecules involved, and that the glycerate formed contained only half the radioactivity of the total glyoxylate entering this sequence but the same quantity as the C_3 compound found as its first step.

Conversion of glycerate into pyruvate

Cell-free extracts of glycollate-grown *Pseudomonas* catalysed the conversion of glycerate into pyruvate (Table 8). The conversion was dependent upon the addition of a catalytic amount of ATP, indicating that the glycerate was probably phosphorylated to form phosphoglycerate and that the ATP was regenerated in the subsequent conversion of phosphoenolpyruvate into pyruvate. The overall reaction is represented by:

$$\underset{\text{glyceric acid}}{CH_2(OH)\cdot CH(OH)\cdot CO_2H} \xrightarrow{ATP} \underset{\text{pyruvic acid}}{CH_3\cdot CO\cdot CO_2H} + H_2O. \qquad \text{(iv)}$$

The rate of the overall process was measured by converting the pyruvate into lactate in the presence of DPNH and crystalline lactic dehydrogenase. Under these conditions 7·2 μmoles of lactate were formed from glycerate/hr./mg. of protein in the extract. No lactate was formed if the extract, glycerate or ATP, was omitted (Fig. 6).

When radioactive glycerate, enzymically prepared by incubating extracts of glycollate-grown *Pseudomonas* with [1-^{14}C]glyoxylate and DPNH [reactions (ii)–(iii)], was used as the substrate for pyruvate formation, radioactive pyruvate was isolated at the end of the reaction (Table 9). This demonstrates the overall conversion of [1-^{14}C]-glyoxylate into radioactive pyruvate, being the sum of reactions (ii)–(iv).

Rates of enzymic reactions in extracts of Pseudomonas (B_2aba)

Sonic extracts of *Pseudomonas* (B_2aba) grown on succinate differed markedly from those grown on glycollate in their ability to catalyse relevant reactions (Table 10). Glycollate was oxidized [reaction (i)] 50 times as fast by extracts of glycollate-grown cells as by those grown on succinate; similarly the rates of the glyoxylate carboligase reaction [reaction (ii)] and the formation of pyruvate from glycerate [reaction (iv)] occurred respectively at at least 50 and 700 times the rates in the former than in the latter extracts. This is evidence for the view that these reactions play a role in the metabolism of glycollate, and are not of importance when glycollate (or glyoxylate) are not major substrates.

Both extracts grown on glycollate and on succinate contained malate synthetase and con-

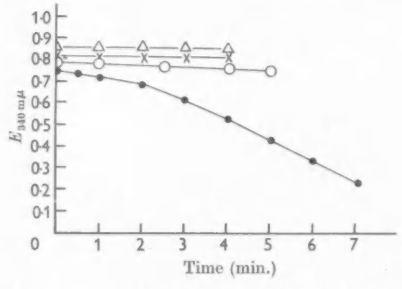


Fig. 6. Conversion of glycerate into lactate catalysed by extracts of glycollate-grown *Pseudomonas* (B_2aba). The complete system (●) contained, in 3 ml., 100 μmoles of tris buffer, pH 7·6, 10 μmoles of $MgCl_2$, 5 μmoles of reduced glutathione, 0·2 μmole of ATP, 2000 units of crystalline lactic dehydrogenase, 0·40 μmole of DPNH and 0·05 ml. of sonic extract of glycollate-grown *Pseudomonas* (B_2aba) (containing 0·35 mg. of soluble protein). The reaction was started by addition of 5 μmoles of sodium D(−)-glycerate and was measured as the change in extinction with time at 340 mμ concomitant with the oxidation of DPNH. Control cuvettes contained the above system but omitting cell extract (△) or ATP (×) or glycerate (○).

Table 9. *Conversion of* [^{14}C]*glycerate into pyruvate*

The complete system contained 0·2 ml. of extract of glycollate-grown *Pseudomonas* (B_2aba), prepared in the Hughes press (Hughes, 1951) and containing 2 mg. of protein, 100 μmoles of potassium phosphate, pH 7·2, 10 μmoles of $MgCl_2$, 5 μmoles of glutathione, 2 μmoles of sodium D(−)-glycerate, approx. 0·5 μmole of [^{14}C]glycerate eluted from chromatograms (see Methods section) and containing $2·5 \times 10^3$ counts/min., 5 μmoles of ATP and water to 1 ml. After incubation for 30 min. at 30°, 2·5 μmoles of sodium pyruvate and 0·2 ml. of 50% (w/v) trichloroacetic acid were added. The precipitate was removed by centrifuging. To the supernatant solution was added 0·2 ml. of saturated 2:4-dinitrophenylhydrazine in 2N-HCl, the 2:4-dinitrophenylhydrazone was extracted and separated by chromatography (El Hawary & Thompson, 1953), and its radioactivity was determined by direct radioassay of the yellow spots with a mica end-window β-counter tube (G.E.C., type 2B2).

Reaction mixture	Radioactivity of pyruvate 2:4-dinitrophenylhydrazone (counts/min.)
(1) Complete system	510
(2) As (1) but ATP omitted	24
(3) As (1) but extract boiled before incubation	0

Table 10. *Enzymic reactions in extracts of* Pseudomonas (B_2aba)

The rates of the reactions in sonic extracts are expressed as specific activities (μmoles of substrate transformed/mg. of soluble protein/hr.). For conditions of assay, see Methods section.

Reaction no.	Enzymic activity	Specific activity in extracts of *Pseudomonas* (B_2aba) grown on: Glycollate	Succinate
(i)	Glycollic oxidase	3·5	0·07
(ii)	Glyoxylate carboligase	41·0	0·80
(iii)	Formation of glycerate from C_3 compound	47·0	—
(iv)	Formation of pyruvate from glycerate	7·2	0·01
(v)	Malate synthetase	104	6·1
(vi)	Malic dehydrogenase	143	157
(vii)	Formation of pyruvate from oxaloacetate	102	130
(viii)	Pyruvate oxidase	1·2	—
—	Condensing enzyme	24·8	42·6
—	*iso*Citratase	0·21	—

densing enzyme (cf. Kornberg & Lund, 1959), but differed in the absolute levels of these enzymes and in their relative amounts. Glycollate-grown cells contained 17 times as much malate synthetase but less than two-thirds as much condensing enzyme as did cells grown on succinate; moreover, the ratio of activities of malate synthetase to condensing enzyme were 4·2:1 for the former and 0·14:1 for the latter extract. These observations indicate the relatively greater importance of malate synthetase and lesser importance of condensing enzyme in the metabolism of glycollate than in that of succinate, and further suggest that in *Pseudomonas* (B_2aba) as in *E. coli*, strain *w* (Kornberg & Sadler, 1960), the oxidation of glycollate proceeds via a dicarboxylic acid cycle. As expected from the known metabolic routes of succinate and those postulated for glycollate, change of carbon source for growth produced no major difference in the rates of formation of pyruvate from oxaloacetate and in the activity of malic dehydrogenase. As had been previously reported for *P. ovalis* Chester (Kornberg, Gotto & Lund, 1958) and *E. coli* (Kornberg & Sadler, 1960), extracts of glycollate-grown *Pseudomonas* (B_2aba) contained negligible *iso*citratase activity.

DISCUSSION

When micro-organisms grow on C_2 compounds as the sole carbon source, reactions must occur whereby the C_2 substrate yields both energy and carbon to the cell. The oxidation of glycollate in *E. coli*, strain *w*, has been shown to be effected, at least in part, via a dicarboxylic acid cycle (Kornberg & Sadler, 1960), in which the component reactions are

$$\text{glycollate} + \tfrac{1}{2}O_2 \rightarrow \text{glyoxylate} + H_2O \quad \text{(i)}$$

$$\text{glyoxylate} + \text{acetyl-coenzyme A} + H_2O \rightarrow \text{malate} + \text{coenzyme A} \quad \text{(v)}$$

$$\text{malate} + \tfrac{1}{2}O_2 \rightarrow \text{oxaloacetate} + H_2O \quad \text{(vi)}$$

$$\text{oxaloacetate} \xrightarrow[\text{phosphopyruvate}]{\text{possibly via}} \text{pyruvate} + CO_2 \quad \text{(vii)}$$

$$\text{pyruvate} + \tfrac{1}{2}O_2 + \text{coenzyme A} \rightarrow \text{acetyl-coenzyme A} + CO_2 + H_2O \quad \text{(viii)}$$

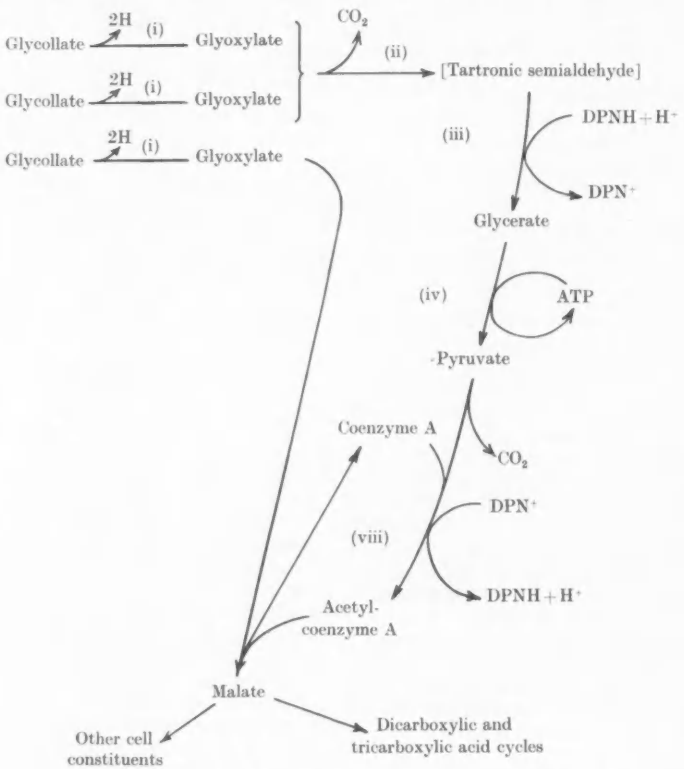
$$\text{Sum: glycollate} + 1\tfrac{1}{2}O_2 \rightarrow 2CO_2 + 2H_2O. \quad \text{(ix)}$$

The observation that isotope from [2-^{14}C]-glycollate was incorporated initially by glycollate-grown *Pseudomonas* (B_2aba) into glycine (presumably by amination of glyoxylate, which is not detected by the chromatographic procedures used) and into malate indicates that this dicarboxylic acid cycle serves also in *Pseudomonas* (B_2aba) to provide metabolic energy from the oxidation of glycollate. This is supported by the finding that the levels of malate synthetase (v) in extracts of *Pseudomonas* (B_2aba) grown on glycollate or on other substrates catabolized chiefly to glyoxylate (such as allantoin) are 4–5 times as high as those of the citrate-forming condensing enzyme, whereas in extracts of the same organisms grown on succinate, or other substrates catabolized chiefly to intermediates of the tricarboxylic acid cycle, the malate-synthetase activity is approx. one-seventh of that of the condensing enzyme; moreover, the absolute levels of condensing enzyme are lower in glycollate-grown cells than in cells grown on substrates catabolized to intermediates of the tricarboxylic acid cycle. This suggests that during the growth of *Pseudomonas* (B_2aba) on glycollate, malate synthetase is involved both in respiration and in the biosynthesis of cellular materials, whereas

condensing enzyme fulfils chiefly the latter function.

Whereas the operation of this dicarboxylic acid cycle serves to supply metabolic energy to the cell from the catabolism of glycollate, it cannot explain growth on glycollate. Cellular constituents, such as carbohydrate and protein, are synthesized by a series of reactions in which intermediates of the dicarboxylic and tricarboxylic acid cycles serve as starting materials (for review, see Roberts, Abelson, Cowie, Bolton & Britten, 1955; Davis, 1955; Ehrensvärd, 1955; Kornberg, 1959). Growth therefore necessarily involves the removal of such intermediates from these cycles, and cannot take place unless reactions occur to effect the net formation of these compounds from glycollate.

The patterns of distribution of isotope incorporated from [2-^{14}C]glycollate by *Pseudomonas* (B_2aba) growing on it indicated that, besides glyoxylate and malate, phosphoglyceric acid was rapidly formed from the C_2 substrate. Glutamate synthesized under similar conditions but in which [1-^{14}C]-glycollate was the substrate, was almost exclusively labelled in the α-carboxyl group (H. L. Kornberg & A. M. Gotto, unpublished work). This latter observation confirms the earlier work of Jayasuriya (1955) who found that approx. 7 times as much isotope was incorporated into citrate from [2-^{14}C]glycollate as from [1-^{14}C]glycollate, and that with the former isotopic substrate, all six carbon atoms of citrate were labelled whereas, with the latter, isotope was incorporated only into two of the three carboxyl groups. Both the isotope distribution observed in brief incubations and the pattern of labelling of citrate and glutamate support the operation of the biosynthetic sequence out-



Scheme 1. Postulated route for the formation of cell constituents from glycollate by *Pseudomonas* (B_2aba). The overall reaction leading from glycollate to malate by this sequence is

$$3 \text{ glycollate} \rightarrow \text{malate} + 2CO_2 + 6H.$$

Roman numbers in parentheses refer to reactions discussed in the text.

lined in Scheme 1. This sequence involves the oxidation of glycollate to glyoxylate (i) and the oxidative condensation of glyoxylate to carbon dioxide and a C_3 compound, the properties of which differed from those of hydroxypyruvate but were similar to those of tartronic semialdehyde (Chow & Vennesland, 1958). The carbon dioxide evolved in this reaction (ii) contains half the isotope from [1-^{14}C]glycollate entering this reaction sequence (Table 4). Reduction of the C_3 compound (probably tartronic acid semialdehyde) yields glyceric acid (iii), which is phosphorylated to phosphoglyceric acid. Since both glyoxylate itself and the tartronic acid semialdehyde are fugitive on chromatograms, it would thus be expected that one of the first-labelled materials formed from added [1-^{14}C]glycollate should be glycine or phosphoglycerate or both, as was observed. The subsequent metabolism of phosphoglycerate is explained by the well-established reactions of the Embden–Meyerhof scheme. Pyruvate or phosphopyruvate thus formed (iv) can react with carbon dioxide to yield oxaloacetate or malate, in which case one-half of the initial isotope would still be retained, or can be oxidized to acetyl-coenzyme A and carbon dioxide, in which case the evolved carbon dioxide would contain the remainder of the isotopic carbon derived from [1-^{14}C]glycollate, and the acetyl-coenzyme A would be unlabelled. Condensation of the acetyl-coenzyme A with another molecule of [1-^{14}C]glyoxylate derived from [1-^{14}C]glycollate would then yield malate, which, if randomized via fumarate, again contains isotope at half the activity of the [1-^{14}C]glycollate but distributed over its two carboxyl groups. Subsequent oxidation of malate to oxaloacetate and its condensation with another molecule of (unlabelled) acetyl-coenzyme A would result in the formation of citrate labelled only in two of its three carboxyl groups, and glutamate labelled exclusively in the α-carboxyl carbon, as was experimentally observed.

Further evidence for the operation of the postulated biosynthetic sequence, all the component reactions of which have been demonstrated to occur in extracts of glycollate-grown *Pseudomonas* at rates of the order necessary to account for the growth of the organisms on glycollate (mean generation time, 3 hr.), is provided by the observation that both the enzymic conversion of glycerate into pyruvate (iv) and glyoxylate carboligase (ii) occur in high activity only in cells grown on substrates the major catabolic product of which is glyoxylate (cf. Gray, Gerhart & Brooke, 1959). The studies of Callely & Dagley (1959) and of Quayle & Keech (1959) indicate that this or a closely similar biosynthetic sequence operates also in *Pseudomonas* growing on glycine or oxalate as sole carbon source.

SUMMARY

1. Washed suspensions of *Pseudomonas* (B_2aba), grown on glycollate, readily oxidized glycollate, glyoxylate, glycerate, pyruvate and malate. Of these substrates, only malate was readily oxidized by the organisms grown on succinate.

2. *Pseudomonas* (B_2aba) growing on glycollate incorporated ^{14}C from [2-^{14}C]glycollate most rapidly into glycine, phosphoglycerate and malate.

3. Extracts of such cells catalysed the oxidation of glycollate to glyoxylate, which was stimulated by phenazine methosulphate.

4. Such extracts further catalysed the anaerobic condensative decarboxylation of glyoxylate to carbon dioxide and a C_3 compound, which differed from hydroxypyruvate and was probably tartronic semialdehyde. This reaction required magnesium ions and thiamine pyrophosphate for maximal activity. One molecule of carbon dioxide was evolved for each two molecules of glyoxylate added; experiments with [1-^{14}C]glyoxylate showed that the evolved carbon dioxide arose from the carboxyl group of one of the glyoxylate molecules reacting.

5. The tartronic semialdehyde formed was enzymically reduced to glyceric acid with the concomitant stoicheiometric oxidation of reduced diphosphopyridine nucleotide.

6. In the presence of magnesium ions, thiamine pyrophosphate and reduced diphosphopyridine nucleotide, such cell extracts catalysed the incorporation of isotope from [1-^{14}C]glyoxylate into carbon dioxide and glyceric acid, this overall reaction being:

$$2\ \text{glyoxylate} + \text{DPNH} + \text{H}^+ \rightarrow \text{glycerate} + \text{CO}_2 + \text{DPN}^+.$$

The quantitative net formation of glycerate from glyoxylate under these conditions was demonstrated.

7. Extracts of glycollate-grown *Pseudomonas* (B_2aba) catalysed the incorporation of isotope from [^{14}C]glycerate into pyruvate, and the net formation of pyruvate from glycerate, if fortified with catalytic quantities of adenosine triphosphate.

8. Such extracts also catalysed the oxidation of pyruvate and the formation of malate from acetyl-coenzyme A and glyoxylate; they contained high activities of condensing enzyme, malic dehydrogenase and oxaloacetic carboxylase(s), but only negligibly small amounts of *iso*citratase.

9. Extracts of glycollate-grown *Pseudomonas* (B_2aba) contained 50 times the activity of glycollic oxidase and glyoxylate carboligase of, and formed pyruvate from glycerate at least 700 times as fast as, similar extracts of succinate-grown *Pseudomonas*

(B_2aba). The former extracts also contained 17 times as much malate synthetase, but less than two-thirds as much condensing enzyme, as the latter.

10. On the basis of these results, a scheme is proposed to account for the biosynthesis of cell constituents from glycollate. It is further concluded that the oxidation of glycollate by *Pseudomonas* (B_2aba) proceeds at least in part via a dicarboxylic acid cycle.

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The Biochemistry of Epithelia: Enzyme Levels in Bronchial and Tracheal Epithelia of Smokers and Non-Smokers and in Bronchial Carcinoma

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Spencer (1958) developed a method for the removal of bronchial epithelium from human autopsy specimens in a form suitable for the estimation of various biochemical entities and allowing a strict cytological basis for their expression. Investigation of the epithelial-cell suspensions so prepared established conditions for the assay of several hydrolytic enzymes and showed the validity of the assays in autopsy material removed up to 6 hr. *post mortem* (Spencer, 1959). The material was stored at $-20°$ with no noticeable decrease in the enzyme activity after 6 months.

The present study was designed to investigate the effect of smoking on the biochemistry of bronchial epithelium. The rationale of the study was to measure various enzymes and substances in suspensions of human bronchial epithelium removed at autopsy and to correlate the routine assays with smoking habits and clinical records in an attempt to detect precancerous 'biochemical lesions' which could be associated with the inhalation of tobacco smoke. Various histological precancerous lesions have already been reported to be more numerous in the tracheobronchial tract of smokers than in non-smokers (Auerbach *et al.* 1957*a*, *b*; Chang, 1957; Ide, Suntzeff & Cowdry, 1959).

The occurrence of carcinoma of the trachea is very low compared with that of the bronchus and no significant rise in incidence of tracheal carcinoma has been observed during the last few years to parallel the increase in bronchial carcinoma (cf. Griswold, Wilder, Cutler & Pollack, 1955), which the majority of epidemiological studies have associated with the smoking habit (for a review see Davies, 1956). Comparison of the bronchial epithelium with the tracheal epithelium from the same subject was therefore made to distinguish between carcinogenic and non-specific effects of tobacco smoke. To avoid confusion between changes due solely to variations in cell population of the epithelia and specific precancerous changes, the biochemical assays were related to the cytological make-up of the epithelial-cell suspensions.

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Further aid to the recognition of cancerous and precancerous changes was sought by comparison of the enzyme levels in tracheobronchial epithelia with those of human-lung squamous-cell carcinomas removed at surgery and human bronchial carcinoma transplants in hamster cheek-pouch. To increase the possiblities of detecting any precancerous changes, epithelial-cell samples were removed from the right main-stem bronchus at a position where the incidence of squamous-cell carcinoma is greatest, and the cases studied were restricted to males over the age of 40 years, the age group in which bronchial carcinoma is most frequent. A number of enzymes, selected for reasons stated by Spencer (1959), have now been studied in this material.

EXPERIMENTAL

Autopsy material. The autopsy specimens considered for analysis were restricted to those from white and coloured American males over the age of 40 years. Within this broad specification considerable care was exercised in the selection of specimens. Contra-indications included the lapse of more than 6 hr. between death and cooling of the removed epithelial-cell suspension to $-20°$; presence of purulent mucus; terminal pneumonia, bronchitis or other respiratory disease; aspiration of gastric contents; extensive haemorrhage; inanition. The specimens examined totalled 87 and only 22 proved to be suitable for the present investigation. Two further cases with terminal pneumonia were also investigated. Of the cases analysed, nine were non-smokers, whereas the others had smoked cigarettes to various degrees and for various lengths of time. In view of the final findings no attempt has been made to classify the smokers into different groups.

Preparation of samples. The epithelium from 2 cm.^{2} of the right main-stem bronchus approx. 2 cm. below the bifurcation was suspended in 1·2 ml. of ice-cold iso-osmotic sucrose (0·25 M) containing 0·01 M-ethylenediaminetetra-acetate (sodium salt) at pH 7·4, by the brush technique of Spencer (1958). The epithelial-cell suspension was immediately distributed in 10, 20 and 40 μl. portions among a number of small tubes, the design of which was dictated by the different enzymes and substances to be examined (Spencer, 1959). The tubes were frozen immediately and stored at $-20°$ until required for assay. Other portions were retained for total and differential cell counts in duplicate. The denuded areas of the bronchus were fixed in Bouin fluid and sectioned, and sections were stained with

haematoxylin and eosin and examined microscopically to check the extent of removal of the epithelium (Spencer, 1958). Removal was complete in all cases. The epithelium from a 2 cm.^{2} area of the trachea 3–5 cm. above the caryna was similarly treated. Portions of the bronchus and trachea contiguous with those removed for biochemical analysis were examined in a cytological study which has been reported elsewhere (Ide *et al.* 1959).

Human bronchial (squamous-cell) carcinoma samples were obtained within 0·5 hr. after surgical removal. Portions about 1 cm.^{3}, which were macroscopically non-necrotic, were placed on a microscope slide and coarsely minced with a scalpel. Pressure on the cut tissue with the flat blade of the scalpel resulted in an exudation of the loosely packed carcinoma cells. The exudate was suspended in 2 ml. of ice-cold 0·25 M-sucrose, mixed by five passages through the orifice of a pipette and allowed to stand for about 2 min. at 0°. The material still in suspension was removed with a pipette, leaving connective tissue at the bottom of the tube. The carcinoma-cell suspension was diluted with cold 0·25 M-sucrose to a concentration of about 6×10^6 cells/ml., which was suitable for the enzyme estimations. The diluted suspension was distributed amongst a number of small tubes as for the epithelial-cell suspensions and then frozen and stored at −20°. Further portions were retained for total and differential cell counts. These preparations showed negligible contamination with connective-tissue cells and leucocyte contamination was below 5%.

Human bronchial carcinoma transplants in the cheek-pouch of cortisone-treated hamsters (Sirota & Wishnow, 1958) were removed 9 days after transplantation and treated in a similar fashion to the surgically removed specimens. The transplanted tumours were highly vascularized and careful dissection of the material was necessary before mincing to restrict the contamination of the final cell suspension by blood cells to below 5%.

Cytoplasmic volumes. Smears of the cell suspensions were stained with Alcian Blue and Neutral Red (Spencer, 1958) and the areas of each of 25 goblet, ciliated, intermediate and basal cells and their respective nuclei, selected at random, were measured with a camera lucida and a planimeter. The cells and nuclei were classified as spheres, prolate spheroids, cylinders or cones, depending on their sectional shape, and their volumes were calculated by multiplying their diameters by the factors 0·67 (spheres), 0·67 (×the smaller diameter, prolate spheroids), 0·79 (cylinders) and 0·52 (×the diameter across the top, cones). After subtraction of the volumes of the individual nuclei the volume of the cytoplasm of each cell and then the average cytoplasmic volume of each type of cell was calculated. From the differential cell count it was possible to calculate the overall average cytoplasmic volume of the cells of the suspension. The final value arrived at was considered only a rough approximation since the cells were never perfect geometrical shapes and since no allowance could be made for the flattening of the cells on the slide or for differential shrinkage between individual cells or even between cytoplasm and nuclei. A further source of error was the space in the theca of the goblet cells, but a partial correction was made by measuring only the volumes of empty goblet cells in which the theca is occupying a minimal volume. With Alcian Blue the mucus was stained blue and full and empty goblet cells could be easily distinguished.

Assay methods. Acid phosphatase, β-glucuronidase, arylsulphatases A and B and arylsulphatase C were assayed under the conditions previously found to be optimum for these enzymes in human bronchial epithelium (Spencer, 1959). Lactic and malic dehydrogenases were assayed by the method of Robins, Roberts, Eydt, Lowry & Smith (1956) in diluted 10 μl. samples of epithelial suspensions. Protein nitrogen was measured in 10 μl. samples by an adaptation (Spencer, 1959) of the method of Nyyar & Glick (1954).

Deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) were measured in samples (20 μl.) by an adaptation of the ultramicro method of Lowry, Roberts, Leiner, Wu & Farr (1954) and incorporating a separation of RNA and DNA by differential akaline hydrolysis (Schmidt & Thannhauser, 1945). To a portion (20 μl.) of the epithelial-cell suspension contained in a tapered tube (50 mm. long, 4·5 mm. internal diam.), cooled to 0°, was added 20 μl. of cold 10% (w/v) trichloroacetic acid. After mixing by holding the tube against a flattened nail rotating at about 1500 rev./min. the tube was allowed to stand for 15 min. at 0°, vibrated again and then centrifuged at 0°. The supernatant was withdrawn with a fine-tipped pipette and 80 μl. of cold ethanol containing 0·1 M-potassium acetate was immediately added. The tube was again vibrated, centrifuged and the supernatant was withdrawn. The precipitate was further successively washed on the centrifuge with 80 μl. of ethanol and 80 μl. of ethanol–ether (1:1, v/v), and then dried *in vacuo* for 1 hr. To the dried precipitate was added 20 μl. of 0·2 N-KOH, the tube was vibrated, capped with Parafilm and incubated for 16–20 hr. at 38°. The sample was then cooled to 0° and 36 μl. of 5% (w/v) perchloric acid was added. The tube was vibrated, centrifuged at 0° and the supernatant was transferred to a round-bottomed tube 50 mm. long, 4·5 mm. internal diam. The precipitate was washed on the centrifuge with a further 20 μl. of 5% perchloric acid and the supernatants were combined (RNA sample). To the precipitate in the original tube was added 30 μl. of 5% perchloric acid, the precipitate was suspended by vibration, the tube was capped tightly with a rubber stopper and heated at 75° for 1 hr. After cooling, the tube was centrifuged and the supernatant was transferred to a round-bottomed tube. The precipitate was washed on the centrifuge with 20 μl. of 5% perchloric acid and the supernatants were combined (DNA sample). The phosphate contents of the RNA and DNA samples were then measured by the method of Lowry *et al.* (1954), with 10 μl. of ashing mixture and 100 μl. of the colour reagent. The estimation was carried out in triplicate together with triplicate water blanks, which were taken through the whole procedure. At a later stage it was found more convenient to use a 40 μl. sample of the cell suspension for nucleic acid determinations. All volumes throughout the procedure were then doubled.

RESULTS

Acid phosphatase. The acid-phosphatase activity per cell varied in the different epithelial samples, the highest value being approximately five times that of the lowest. A similar widespread divergence was shown when the activities were calculated on the basis of the DNA contents of the cell suspen-

sions or on their protein-nitrogen contents. There was no significant correlation between enzyme activity and the number of goblet, ciliated or basal cells in the sample, but a rough correlation was seen when the acid-phosphatase activities were plotted against the ratio columnar (ciliated, goblet and intermediate) : basal cells (Fig. 1). In normal human bronchial epithelia this cell ratio is approximately 1:1 (Spencer, 1958), and ratios of the order of 0·5:1 indicate a considerable degree of basal-cell hyperplasia. Ratios greater than 1:1 indicate an increased number of goblet and ciliated cells and also that these columnar cells are generally enlarged (cf. Ide *et al.* 1959). Since the columnar cells are much larger than basal cells the columnar:basal cell ratio is a rough guide to the average size of the cells in the different cell suspensions, and this suggested that the acid-phosphatase activities of the epithelial-cell suspensions might be a reflexion of the relative sizes of the cells. The nuclei of the basal and columnar cells are of similar size and the difference between the two types of cell lies in the large cytoplasmic volume of the latter type. RNA/DNA ratios are generally accepted to be a useful measure of cytoplasmic:nuclear ratios and, as shown below, there was good correlation between the RNA content and the cytoplasmic volume of the epithelial-cell suspensions. Accordingly the acid-phosphatase activities were plotted against the average RNA content/cell.

A considerable degree of correlation was observed (Fig. 1). Most enzyme values lay within one standard error (drawn to the *Y* axis) of the calculated regression line, whether the activities were those of bronchial or tracheal epithelia or whether the epithelia were derived from smokers or non-smokers. The regression line passed through zero and had a correlation coefficient of 0·94. The acid-phosphatase activities of the five cell suspensions of surgically removed lung carcinomas and the two values for the human bronchial carcinoma transplants in hamster cheek-pouch were reasonable fits to the regression line (Fig. 1).

Other enzymes. Findings almost identical with those for acid phosphatase were made with β-glucuronidase, arylsulphatases A plus B, arylsulphatase C, lactic dehydrogenase and malic dehydrogenase (Figs. 2–6), although in no case was the correlation coefficient as high as for acid phosphatase. The enzyme activities of the human bronchial carcinomas and transplants in hamster were reasonable fits to the respective regression lines. Only one exception was noted; the arylsulphatase C values for bronchial carcinoma transplants in hamster were about five to seven times as high as was expected from their RNA content (Fig. 4). With lactic and malic dehydrogenases the

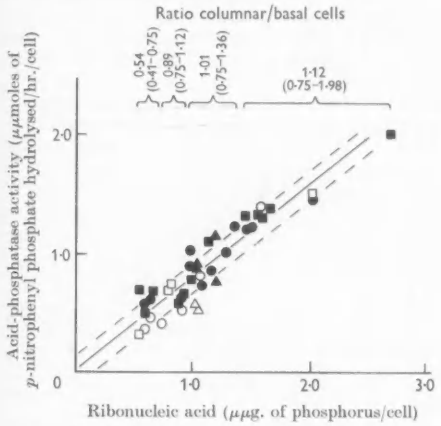

Fig. 1. Acid-phosphatase activity of epithelial-cell suspensions plotted against ribonucleic acid content. The regression line (correlation coefficient 0·94) is drawn as a continuous line and broken lines are drawn at a distance equal to one standard error of the estimate (*Y* axis). □, Bronchus, non-smokers; ■, bronchus, smokers; ○, trachea, non-smokers; ●, trachea, smokers; ▲, bronchial carcinoma, surgical specimens; △, bronchial carcinoma, transplants in hamster cheek-pouch. The average columnar/basal cell ratio is stated with the range in parentheses.

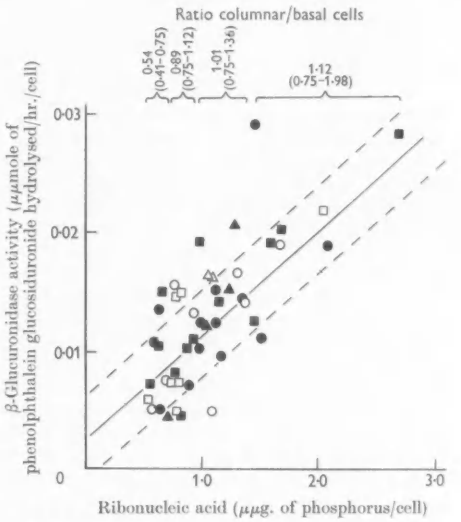

Fig. 2. β-Glucuronidase activity of epithelial-cell suspensions plotted against ribonucleic acid content. Details were as given in Fig. 1. The correlation coefficient of the regression line is 0·76.

slopes of the regression lines were shallow and did not pass within one standard error of the origin. The slope of the regression line for lactic dehydrogenase (0·41) had a standard error of 0·111 and was significantly different from the horizontal ($P < 0·01$).

Ribonucleic acid content and cytoplasmic volume. Although a small amount of RNA is found in nuclei of mammalian cells (Davidson, 1950), the major portion is known to be associated with the endoplasmic reticulum of the cytoplasm. Fig. 7 shows that in the selected samples of bronchial epithelial-cell suspensions there was a reasonable correlation between the average cytoplasmic volume of the cells and their RNA content. This suggests that the enlarged size of the columnar cells, seen in various circumstances, particularly in the epithelia of mild smokers (Ide *et al.* 1959), is due to an increase in the amount of functioning cytoplasm rather than to oedema or other non-specific changes.

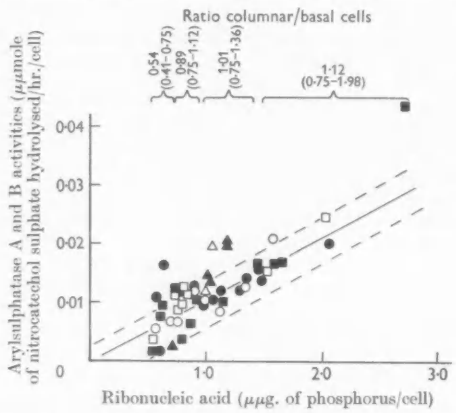

Fig. 3. Arylsulphatases A plus B activity of epithelial-cell suspensions plotted against ribonucleic acid content. Details were as given in Fig. 1. The correlation coefficient of the regression line is 0·84.

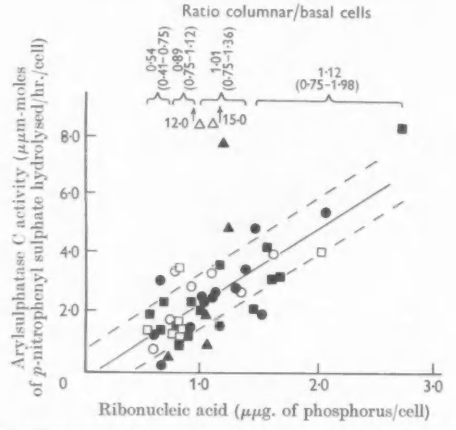

Fig. 4. Arylsulphatase C activity of epithelial-cell suspensions plotted against ribonucleic acid content. Details were as given in Fig. 1. The correlation coefficient of the regression line is 0·80.

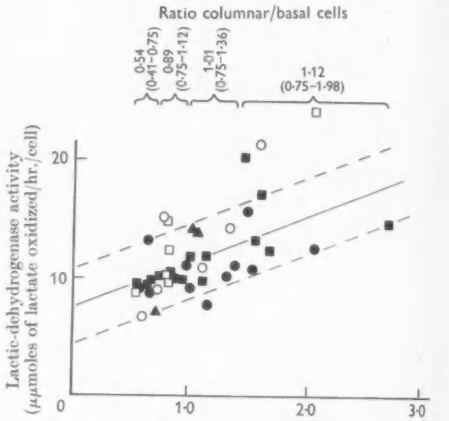

Fig. 5. Lactic-dehydrogenase activity of epithelial-cell suspensions plotted against ribonucleic acid content. Details were as given in Fig. 1. The correlation coefficient of the regression line is 0·54.

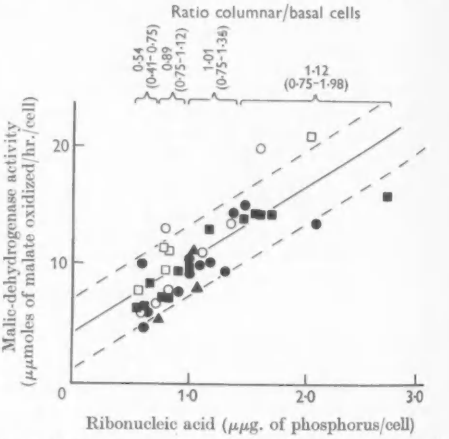

Fig. 6. Malic-dehydrogenase activity of epithelial-cell suspensions plotted against ribonucleic acid content. Details were as given in Fig. 1. The correlation coefficient of the regression line is 0·76.

Deoxyribonucleic acid. Leuchtenberger, Leuchtenberger & Doolin (1958), using ultraviolet microscopy, have measured differences between the DNA values of individual normal mouse bronchial epithelial cells and metaplastic epithelia induced by exposure to tobacco smoke. In the present investigation the DNA modal values in $\mu\mu$g. of phosphorus/cell were: bronchus, non-smokers, 0·65; bronchus, smokers, 0·72; trachea, non-smokers, 0·71; trachea, smokers, 0·72. Statistically these values were the same.

DISCUSSION

Since RNA is almost exclusively localized in the cytoplasm of cells it can be reasoned that when the RNA/enzyme-activity regression line (Figs. 1–6) is extrapolated to zero RNA, the indicated enzyme activity will represent that due to the cell nuclei (Fig. 7). With the enzymes acid phosphatase, β-glucuronidase, arylsulphatases A plus B and arylsulphatase C the regression lines all passed within one standard error of the origin of the axes (Figs. 1–4), and the nuclear activity of these enzymes can therefore be considered to be negligible. The cytoplasmic localization of these enzymes in human tracheobronchial epithelial cells is in accord with the results obtained for the same enzymes in rat-liver cells by the technique of differential centrifuging of cell homogenates. Although a good correlation between RNA content of the cells and enzymes which are localized in the endoplasmic reticulum might have been expected, a similar high degree of correlation was seen with acid phosphatase and arylsulphatases A plus B, which are found in the lysozymes (Viala & Gianetto, 1955; Dodgson, Spencer & Thomas, 1954), and β-glucuronidase, which is of mixed mitochondria–lysozyme localization (de Duve, Pressman, Gianetto, Wattiaux & Appelmans, 1955). The correlation of RNA content with the activities of these various enzymes may indicate some direct relationship, or, both the RNA content and the enzyme activities may simply be reflexions of the variation in cytoplasmic size of the cell (Fig. 7). In either case the cells can be considered to possess a remarkable ability to maintain a cytoplasm of constant composition as far as the components examined are concerned.

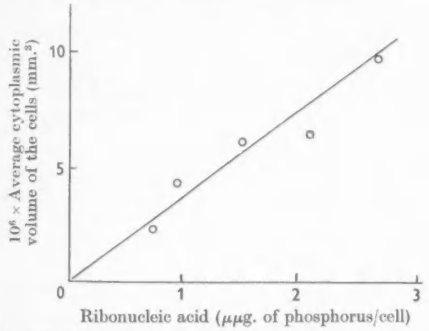

Fig. 7. Variation of the average cytoplasmic volume of cells of bronchial epithelia with their ribonucleic acid content.

At least two interpretations are possible in those cases (lactic and malic dehydrogenases, Figs. 5 and 6) where the RNA/enzyme-regression line cuts the abscissa at a point more than one standard error away from zero enzyme activity. The first is that the activity indicated on the abscissa is that of the nuclei of the cells. The second interpretation invokes the presence of two sources of the enzyme in the cytoplasm: one source containing a fixed and irreducible amount of the enzyme regardless of cell size whereas the amount of enzyme in the other source would vary with the amount of cytoplasm. Lactic and malic dehydrogenases are known to be localized exclusively in the cytoplasm of the liver and heart-muscle cells of rats, and, since amongst mammals the intracellular localization of a particular enzyme is usually the same in all tissues and even in all species, it would seem that the second interpretation of the regression lines for lactic and malic dehydrogenases of human tracheobronchial epithelia is the more probable. Some support for the rather tenuous suggestion that the epithelial cells have two sources of each dehydrogenase in their cytoplasm is provided by the recent demonstration of two individual cytoplasmic malic dehydrogenases in rat liver (Delbruck, Schimassek, Bartsch & Bucher, 1959; Thorne, 1960).

The cytological composition of the specimens used in the calculation of the regression lines varied widely: the extremes were, on the one hand, two cases showing extensive basal-cell metaplasia and two cases showing extensive squamous metaplasia (columnar/basal cells, 0·5), and, on the other hand, several cases, including one with pneumonia, showing increased numbers of goblet and ciliated cells (columnar/basal cells, 1·5). It is concluded from the high correlation coefficients of the regression lines that acid phosphatase, β-glucuronidase, arylsulphatases A plus B, arylsulphatase C and lactic and malic dehydrogenases are not therefore specifically involved in the differentiation of basal epithelial cells into columnar or squamous cells and that these enzymes are concerned in the general metabolism rather than in any specialized function of the epithelial cells. This implies that none of the enzymes mentioned is directly concerned in cilia action or in mucus production, and this implication was borne out by the lack of correlation of any of the enzyme activities with the number of ciliated or goblet cells in the various

specimens. The origin of bronchial carcinomas is the basal cells of the bronchial epithelium, and the finding that the enzyme activities of the five surgically removed carcinomas and the two transplants in hamster cheek-pouch fit reasonably well on the regression lines strongly suggests that the enzymes examined are not specifically involved in the differentiation of the normal basal cells to neoplastic cells. It is further significant that, in the calculation of the regression lines, the enzyme activities of the epithelia from both the trachea and bronchus were included. The values for these samples (Figs. 1–6) suggest that the effect of tobacco smoke on the enzymes in the epithelia is the same in both trachea and bronchus and that this represents a non-specific rather than a carcinogenic effect of tobacco. This finding parallels the cytological studies (Ide *et al.* 1959), which have shown that smoking brings about the same cytological changes in both bronchial and tracheal epithelia.

The basis of the choice of enzymes for testing this method of study was mainly that of expediency (Spencer, 1959), and it was unfortunate that none of the enzymes proved to be of direct interest as far as bronchial carcinoma was concerned. Some of these enzymes have been said by others, on the basis of various experimental results, either to rise or fall in carcinoma when compared with the tissue of origin. These conclusions have been drawn with various parameters for the expression of enzyme activities such as wet weight of tissue or nitrogen content. However, as shown in this study, no difference was seen between normal and tumour tissue when the enzyme activities were placed on a cytoplasmic basis. It is suggested that in biochemical studies on tumours more emphasis should be placed on cytological control of the samples examined.

SUMMARY

1. Epithelia from the bronchus and trachea of human males over the age of 40 were removed within 6 hr. *post mortem* and stored at $-20°$ as suspensions in 0·25 M-sucrose containing 0·01 M-ethylenediaminetetra-acetate, pH 7·4. Clinical histories and smoking habits of the subject were noted.

2. The acid-phosphatase, β-glucuronidase, arylsulphatases A plus B, arylsulphatase C and lactic- and malic-dehydrogenase activities of these suspensions were assayed and correlated with total and differential cell counts, protein nitrogen and deoxyribonucleic acid and ribonucleic acid contents.

3. The activities of all the enzymes showed good correlation with the ribonucleic acid content of the epithelial cells, the regression lines having correlation coefficients of between 0·54 and 0·94. There was no significant correlation between enzyme activities and any of the other parameters.

4. The enzyme activities of suspensions of surgically removed bronchial carcinomas (five specimens) and human bronchial carcinoma transplants in hamster cheek-pouch (two specimens) fitted reasonably well on the regression lines.

5. Analysis of these results suggested that all the enzymes examined are of cytoplasmic occurrence, that they are not directly involved in cilia action or mucus production and that they are not specifically involved in the differentiation of the basal epithelia cells into columnar, squamous or neoplastic cells.

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The Metabolism of Isolated Rat-Liver Nuclei

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The investigations described in this paper were undertaken as a preliminary to the study of the biochemical changes in the nucleus of the rat-liver cell during the induction of liver cancer. Allfrey (1954) and Stern & Mirsky (1953) described the isolation in aqueous media of nuclei from the thymus gland of the calf. Such preparations *in vitro* incorporated glycine and other amino acids into nuclear proteins (Allfrey, Mirsky & Osawa, 1955), and orotic acid and adenosine into the ribonucleic acid fraction of the nucleus (Allfrey & Mirsky, 1957). No comparable studies have been carried out on rat-liver nuclei. However, Logan, Ficq & Errera (1959), using radioautographic techniques, have shown that isolated rat-liver nuclei will take up phenylalanine and adenine *in vitro*.

There is considerable doubt about the mechanisms whereby the nucleus obtains its energy supply. Evidence so far obtained points to some form of oxidative phosphorylation (Allfrey & Mirsky, 1957), but the nature of the oxidative pathway involved is still unknown.

In this paper the chemical composition of isolated rat-liver nuclei has been determined, and protein and nucleic acid syntheses have been investigated. The effects of anoxia, 2:4-dinitrophenol, chlorpromazine and azide on these reactions have been studied. Investigations of the adenosinetriphosphatase activities of the preparations coupled with the results of the synthetic experiments have thrown further light on the underlying energy mechanisms of the nucleus.

MATERIALS AND METHODS

Animals. Male hooded Norwegian rats, fed on the M.R.C. 41B diet without restriction, were used, weight 200–250 g.

Reagents. Glass-distilled water was used throughout. Cytochrome *c*, adenosine 5′-triphosphate (ATP) and reduced diphosphopyridine nucleotide (DPNH) were obtained from Sigma Chemical Co., St Louis, Mo., U.S.A. Neotetrazolium chloride, sodium azide and dicoumarin were obtained from L. Light and Co. Ltd., Colnbrook, Bucks., England. Chlorpromazine was a gift from May and Baker Ltd., Dagenham, Essex. ^{32}P, [2-^{14}C]glycine, [6-^{14}C]-orotic acid and [8-^{14}C]adenine hemisulphate were obtained from The Radiochemical Centre, Amersham, Bucks. ^{32}P was purified as described by Kennedy (1953).

Tissue preparations. Mitochondria were isolated from rat liver in 0·25 M-sucrose by the method of Schneider (1948). Soluble cytoplasm (cell sap) was prepared from rat liver in 0·25 M-sucrose as described by Zamecnik & Keller (1954).

For the preparation of nuclei a rat was anaesthetized with ether, and the liver was perfused *in situ* through the portal vein with 20 ml. of ice-cold 0·9% (w/v) sodium chloride solution. The inferior vena cava was severed, and the perfusion was continued with 100 ml. of ice-cold 0·25 M-sucrose containing calcium chloride to a final concentration of 1·8 mM. This procedure freed the liver of nearly all the erythrocytes. The liver was removed, weighed and homogenized in 50 ml. of ice-cold 0·25 M-sucrose containing calcium chloride to a final concentration of 5 mM with about 12 strokes in a vertical hand-operated homogenizer with a nylon sphere pestle [modification of the apparatus described by Dounce (1955)]. The homogenate was filtered in the cold through nylon bolting cloth of two mesh sizes (60 and 110 mesh/in.). The filtrate was made to form a layer with an equal volume of ice-cold 0·34 M-sucrose containing a final concentration of 0·2 mM-calcium chloride and centrifuged at 8200 *g* (9000 rev./min.) for 2 min. at 3° in a MSE Angle 13 refrigerated centrifuge. The supernatant was removed, and the loose sediment was resuspended in half the original volume of ice-cold 0·25 M-sucrose, made to form a layer with an equal volume of ice-cold 0·34 M-sucrose and centrifuged at 4800 *g* (7000 rev./min.) for 2 min. at 3°. This procedure was repeated three times more with centrifuge speeds of 2400 *g* (5000 rev./min.), 1600 *g* (4000 rev./min.) and 1000 *g* (3000 rev./min.). The final residue was suspended in 10 ml. of ice-cold 0·25 M-sucrose. A microscopic examination was made of all preparations to ensure freedom from contamination by cellular debris and erythrocytes. Counts of nuclear numbers were made as described by Grant & Rees (1957).

Analytical methods

Breakdown of ATP was studied in the system: ATP, 5 mM; $MgSO_4$, 10 mM; KCl, 37·5 mM; 2-amino-2-hydroxymethylpropane-1:3-diol (tris)–HCl buffer, pH 7·2, 50 mM; 0·5 ml. of nuclear suspension and water to 2·0 ml. The system was incubated at 28° for 20 min. The inorganic phosphate liberated was determined by the method of Fiske & Subbarow (1925).

Deoxyribonucleic acid (DNA) was determined by the method of Dische (1955). Determination of DPNH–cytochrome *c* reductase was by the method described by Dawkins, Judah & Rees (1959). The DPNH–neotetrazolium-reductase system was determined by the method described by Slater (1959). Ribonucleic acid (RNA) and phospholipid were determined as described by Davidson, Frazer & Hutchinson (1951). Nitrogen was determined by the micro-Kjeldahl method.

Table 1. *Recovery of nuclei from rat-liver homogenates*

Results are expressed per 100 mg. wet wt. of liver. Results are the means of ten concordant experiments.

	Rat-liver homogenate	Nuclear preparation	Recovery (%)
Nuclei (no.)	17×10^5	8×10^5 ($7{\cdot}2 \times 10^5$–$9{\cdot}6 \times 10^5$)	47 (42–56)
Nitrogen (mg.)	3·19	0·164 (0·151–0·179)	5·2 (4·7–5·6)
DNA phosphorus (μg.)	21	9·8 (9·0–12·2)	46 (43–58)

Cytochrome oxidase was measured manometrically in the following system: sodium phosphate buffer, 16 mM, pH 7·4; cytochrome *c*, 167 μM; ascorbate, 10 mM; ethylenediaminetetra-acetic acid (EDTA), 0·3 mM; 0·25 ml. of nuclear suspension in 0·25 M-sucrose (frozen and thawed twice); final volume, 3 ml. The temperature of the bath was 38°. The EDTA was added to decrease the metal-catalysed auto-oxidation of the ascorbate.

Succinoxidase was determined manometrically in the following system: sodium phosphate buffer, 33 mM, pH 7·4; sodium succinate, 33 mM; cytochrome *c*, 33 μM; 0·5 ml. of nuclear suspension in 0·25 M-sucrose solution (frozen and thawed twice); final volume, 3 ml. The temperature of the bath was 38°.

Incorporation of radioactivity in vitro

Incorporation of ^{32}P into RNA was based on the method of Siebert, Lang, Lucius & Rossmüller (1953). The system used was: tris–HCl buffer, pH 7·2, 20 mM; KCl, 75 mM; $MgSO_4$, 5 mM; 1·0 ml. of nuclear suspension in 0·25 M-sucrose; ^{32}P, 0·4 mM with an activity of 5 μC; water to 3·0 ml. The temperature was 30°. The reaction was stopped by the addition of 2·0 ml. of 20% (w/v) trichloroacetic acid. The RNA was extracted from the residue and purified by paper electrophoresis to remove inorganic phosphate by the method of Graymore (1956). Radioactivity was determined by elution of the paper with water and counting the solution in a liquid counter (20th Century Electronics Ltd., thin-walled B6). Measurements are corrected for isotope decay, background and dead time of the instrument.

For incorporation of [6-^{14}C]orotic acid and [8-^{14}C]adenine into RNA the system used was: sodium phosphate buffer, pH 7·4, 20 mM; glucose, 12 mM; NaCl, 10 mM; 1 μC of [^{14}C]adenine or [^{14}C]orotic acid; 1·5 ml. of nuclear suspension in 0·25 M-sucrose; water to 2·5 ml. The temperature was 30°. The reaction was stopped by the addition of 2·0 ml. of ice-cold 10% (w/v) perchloric acid. The precipitate was washed four times with 2·0 ml. of ice-cold 2% (w/v) perchloric acid, twice with ethanol and twice with ether. The radioactivity of the dried protein was determined. In experiments where indicated in the text, the RNA was extracted, purified and hydrolysed (Graymore, 1956). The hydrolysate was run on Whatman no. 3 MM paper between water-cooled plates in 0·05 M-citrate buffer (pH 3·5) at 6 kV and 50 mA for 40 min. The nucleotide areas were identified by viewing in ultraviolet light. Each compound was eluted from the paper with water, and the eluate was dried on a plastic planchet of surface area 1 cm.^{2} for determination of radioactivity.

For incorporation of [2-^{14}C]glycine into nuclear protein the system used was: sodium phosphate buffer, pH 7·4, 20 mM; NaCl, 20 mM; [^{14}C]glycine, 1 μC; 1·5 ml. of nuclear suspension in 0·25 M-sucrose; water to 2·5 ml. The temperature was 38°. The reaction was stopped by the addition of 2·0 ml. of 20% trichloroacetic acid. The precipitate was extracted with 5 ml. of 5% (w/v) trichloroacetic acid for 20 min. at 90°. The protein residue was then washed successively with 5 ml. of 5% trichloroacetic acid containing 1% (w/v) of glycine and 5 ml. of 5% trichloroacetic acid, then twice with ethanol and twice with ether. The protein residue was dried at room temperature.

Table 2. *Chemical composition of rat-liver nuclei isolated in* 0·25 M-*sucrose*

Results are the means of ten concordant experiments.

	$\mu\mu$g./nucleus	μg./mg. of nuclear nitrogen
Nitrogen	164 (153–171)	—
Phosphorus		
DNA	690 (600–760)	61 (53–70)
RNA	670 (580–720)	44 (42–48)
Phospholipid	590 (550–740)	37 (34–44)

Preparation of dried protein residues for measurement of radioactivity

The protein residue was ground in a pestle and mortar, and then compressed with a stainless-steel pestle on a planchet of 1 cm.^{2} surface area. All samples were counted for 5 min. in a 20th Century Electronics gas-flow counter, Type WF2. The results were corrected for background and adjusted for infinite thickness.

RESULTS

Chemical composition

The yield of nuclei from rat liver was followed by counting the nuclei and by determination of the DNA and nitrogen content, both in the homogenate and in the nuclear preparations. Table 1 gives the mean result of ten experiments. The DNA, RNA, nitrogen and phospholipid contents of such nuclear preparations are given in Table 2.

Synthetic reactions

The nuclear preparations incorporated [2-^{14}C]-glycine into nuclear proteins, and [6-^{14}C]orotic acid, [8-^{14}C]adenine and ^{32}P into nuclear RNA *in vitro* (Table 3). The rate of incorporation of all these compounds was approximately linear over 90 min.

In the above experiments with [^{14}C]orotic acid and [^{14}C]adenine, the radioactivity of the dried nuclear protein had been determined. To verify that these substances had been used in true syn-

Table 3. *Incorporation of* [2-^{14}C]*glycine* in vitro *into nuclear protein and of* [6-^{14}C]*orotic acid,* [8-^{14}C]*adenine and* ^{32}P *into nuclear ribonucleic acid*

System used was as described in the Materials and Methods section. Results are those of a representative experiment of a group of four similar experiments.

Time of incubation (min.)	^{32}P (counts/min./μg. of RNA P)	Counts/min. at infinite thickness		
		Glycine	Adenine	Orotic acid
30	34	63	57	48
60	77	92	115	97
90	108	151	181	145

Table 4. *Distribution of* ^{14}C *in nucleotide fractions of ribonucleic acid after incorporation* in vitro

Purification of RNA, hydrolysis and electrophoretic separation of nucleotides were as described in the Materials and Methods section. Results are those of a representative experiment of a group of four similar experiments.

Source of ^{14}C	Counts/min. at infinite thickness				
	Hydrolysed RNA used	Cytidylic acid	Adenylic acid	Guanylic acid	Uridylic acid
[^{14}C]Adenine	117	0	52	2	6
[^{14}C]Orotic acid	98	21	7	9	44

Table 5. *Effects of adenosine triphosphate and cell sap on the incorporation of* [^{14}C]*glycine* in vitro *into nuclear protein*

System was as described in the Materials and Methods section. Results are those of a representative experiment of a group of three similar experiments.

Additions	Counts/min. at infinite thickness	Inhibition (%)
Nil	467	—
ATP (10 μmoles)	287	38·5
Cell sap (0·5 ml.)	318	32·0
Cell sap (0·5 ml.) + ATP (10 μmoles)	279	30·0

Table 6. *Effect of anoxia, azide and 2:4-dinitrophenol on the incorporation of glycine into nuclear protein and of adenine and orotic acid into nuclear ribonucleic acid*

Systems were as described in the Materials and Methods section. Additions are recorded as final concentrations. Incubation time was 90 min. Results are those of a representative experiment of a group of four similar experiments.

Additions or treatment	Counts/min. at infinite thickness		
	Glycine	Adenine	Orotic acid
None	185	181	143
Dinitrophenol			
0·1 mM	132	—	118
0·3 mM	94	102	104
0·6 mM	75	82	70
1·0 mM	55	53	51
Azide			
0·6 mM	70	—	48
0·9 mM	56	8	41
Anoxia	83	61	22

thesis of RNA, the nuclear RNA was isolated, purified and hydrolysed. The hydrolysate was subjected to paper electrophoresis, and the constituent nucleotides were separated and counted. The results of such an experiment are given in Table 4. With orotic acid, the highest activity was in the uridylic acid fraction, and some was also found in cytidylic acid. With adenine by far the greatest activity was in the adenylic acid fraction. In the ^{32}P-incorporation experiments, the RNA was isolated and purified by electrophoresis.

Allfrey *et al.* (1955) demonstrated that replacement of sodium ions by potassium ions in the incubation medium decreased the incorporation of [^{14}C]glycine into thymus nuclear protein. Similarly, Breitman & Webster (1959) found decreased incorporation of glycine and alanine into RNA, DNA and protein of thymus nuclei, but the incorporations of formate, adenine, guanine and adenosine were unimpaired. In view of these findings, the replacement of sodium ions by potassium ions in the incubation medium was examined in the incorporation of [2-^{14}C]glycine into protein and [6-^{14}C]orotic acid into RNA by isolated liver-cell nuclei, but was found to be without effect.

The incorporation of amino acids *in vitro* by microsomal preparations is considerably increased by the addition of cell sap, and by cell sap plus ATP or an ATP-generating system (Zamecnik & Keller, 1954). In the nuclear preparations such additions proved inhibitory but the effects were not additive (Table 5).

Allfrey *et al.* (1955) have suggested that the energy system of thymus nuclei depends on oxidative mechanisms. The effect of anoxia was tested

on the synthesis of RNA and protein by liver-cell nuclei, and the results are given in Table 6. Anoxia decreased the incorporation of glycine into protein by about 40%, the incorporation of adenine by about 60% and that of orotic acid by about 90%.

The action of 2:4-dinitrophenol and of sodium azide was investigated on protein and RNA synthesis (Table 6). The degree of inhibition rose with increasing concentrations of azide and 2:4-dinitrophenol. The latter at a final concentration of 1·0 mM gave a 60% inhibition of protein and RNA synthesis.

Since both dinitrophenol and azide are inhibitors of oxidative phosphorylation, such results may indicate that the energy system in the nucleus is similar to the oxidative phosphorylation mechanisms of the mitochondrion. Chlorpromazine has been shown to inhibit some of the phosphorylation steps associated with oxidative phosphorylation (Dawkins *et al.* 1959). This compound inhibited both protein and RNA synthesis by liver-nuclear preparations (Table 7).

Investigations of energy mechanisms

The inhibitory effects of chlorpromazine, azide, 2:4-dinitrophenol and anoxia on the synthetic reactions suggest the presence of an oxidative phosphorylation mechanism in the nucleus. Extraction of normal liver-nuclei preparations with ice-cold 2% perchloric acid did not reveal the presence of acid-soluble nucleotides.

Liver-nuclei preparations did not incorporate ^{32}P into added ATP, and so an alternative approach was made to study the energy mechanisms of the nuclei by examining the properties of the nuclear adenosine triphosphatase.

There is much evidence to suggest that the magnesium-activated adenosine triphosphatase of mitochondria is a reversal of some of the terminal steps of oxidative phosphorylation in preparations no longer able to couple electron transport with the phosphorylation of adenosine diphosphate (ADP) (Siekevitz, Löw, Ernster & Lindberg, 1958). Schneider (1946) found that 27% of the adenosine triphosphatase of rat-liver homogenate was in the nuclear fraction. Such nuclear fractions were by virtue of their method of preparation contaminated with cell-wall debris, whole cells and possibly mitochondria. An examination of nuclei isolated by the technique given in this paper revealed the presence of an adenosine triphosphatase which is stimulated by magnesium (Table 8). Also included for comparison are results for mitochondrial adenosine triphosphatase.

The effect of azide, dinitrophenol and chlorpromazine on the magnesium-activated adenosine triphosphatase of the nuclei was determined (Table 9). Dinitrophenol stimulated breakdown of ATP by about 30%, and this effect was abolished by azide and chlorpromazine. Since similar results have been obtained with mitochondrial preparations (Dawkins, Judah & Rees, 1960), the possibility of mitochondrial contamination in the nuclear preparation was investigated. Although no mitochondria could be found on microscopic

Table 7. *Effect of chlorpromazine on the incorporation of glycine into nuclear protein and orotic acid and adenine into nuclear ribonucleic acid*

System was as described in the Materials and Methods section; incubation time, 90 min. Additions are recorded as final concentrations. Results are those of a representative experiment of a group of three similar experiments.

	Counts/min. at infinite thickness		
Additions	Glycine	Adenine	Orotic acid
None	400	173	250
Chlorpromazine			
0·15 mM	157	137	208
0·3 mM	78	53	70

Table 8. *Comparison of adenosine-triphosphatase activity of nuclei and mitochondria in 0·25 M-sucrose*

Medium: ATP, 5 mM; $MgSO_4$, 10 mM; KCl, 37·5 mM; tris buffer, pH 7·2, 50 mM. Nuclei and mitochondria were added in 0·5 ml. of 0·25 M-sucrose. Final volume was 2·0 ml.; incubation was for 20 min. at 25°. Dinitrophenol was added to 0·03 mM final concentration. Results are the means of six experiments and the ranges are given.

	P liberated (μmoles/mg. of N/20 min.)	
	Mitochondria	Nuclei
Medium	1·3 (1·1–1·5)	5·3 (4·5–5·6)
Medium – Mg^{2+} ions	1·0 (0·9–1·1)	3·2 (3·0–3·5)
Medium + dinitrophenol	5·1 (4·5–5·3)	7·2 (6·2–7·6)

Table 9. *Action of azide, 2:4-dinitrophenol and chlorpromazine on the adenosine triphosphatase of nuclei in 0·25 M-sucrose*

The medium and conditions were as in Table 8. Nuclei were added in 0·5 ml. of 0·25 M-sucrose. Results are the means of six experiments and the ranges are given. Additions are recorded as final concentrations.

Additions	P liberated (μmoles/mg. of N/20 min.)
Nil	5·3 (4·5–5·6)
Dinitrophenol, 0·03 mM	7·2 (6·2–7·6)
Chlorpromazine, 0·2 mM	5·1 (4·2–5·4)
Azide, 1·0 mM	4·3 (4·0–4·4)
Dinitrophenol, 0·03 mM + chlorpromazine, 0·2 mM	4·4 (4·1–4·7)
Dinitrophenol, 0·03 mM + azide, 1·0 mM	5·3 (4·7–5·6)

Table 10. *Cytochrome-oxidase and succinoxidase activity of nuclear and mitochondrial preparations*

Mitochondria and nuclei suspensions in 0·25 M-sucrose were frozen and thawed twice before use. Results are the means of six experiments and the ranges are given.

Preparation	Succinoxidase activity (μl. of O/mg. of N/hr.)	Cytochrome-oxidase activity (μl. of O/mg. of N/hr.)	Cytochrome oxidase/ succinoxidase
Mitochondria	2000 (1800–2100)	5000 (4800–5300)	2·5
Nuclei	110 (100–135)	535 (520–570)	4·8

examination, the succinoxidase activity of the nuclear preparations was determined, and a small but definite activity was found (Table 10). Succinoxidase is a solely mitochondrial enzyme system (Schneider, 1946). Assuming mitochondrial contamination, the succinoxidase activity found in nuclei indicates the presence of 0·075 mg. of mitochondrial nitrogen in each 1·0 mg. of nuclear nitrogen. The magnesium-activated adenosine triphosphatase equivalent to 0·06 mg. of mitochondrial nitrogen is 0·1 μmole of phosphate liberated/20 min., and in the dinitrophenol-stimulated system it is 0·3 μmole of phosphate. Thus after correction for mitochondrial contamination, the nuclear magnesium-activated adenosine-triphosphatase activity in Table 8 is 5·2 μmoles of phosphate and 6·9 μmoles of phosphate in the dinitrophenol-activated system.

If the energy system of the nucleus is similar to that of the mitochondrion, the phosphorylation reactions would be coupled to an electron-transport system. The inhibitory effects of chlorpromazine on synthetic reactions suggest that a flavoprotein is involved, e.g. DPNH–cytochrome *c* reductase (Dawkins *et al.* 1959, 1960; Siekevitz *et al.* 1958). The nuclear preparations were assayed for this enzyme which was found to be present [7·8 (7·1–8·8) μmoles of cytochrome *c* reduced/hr./mg. of nuclear N]. The presence of this enzyme was confirmed by the finding that nuclear preparations could couple the oxidation of DPNH with the electron acceptor neotetrazolium [15·9 (14·9–17·0) mg. of formazan formed/hr./mg. of nuclear N]. The nuclear activity of this system was about 50 % of that of the mitochondrial activity. This reaction was inhibited by dicoumarin, as was the reaction in the mitochondrial system (Slater, 1959). This suggests that a quinone such as ubiquinone is involved in the oxidation of DPNH. The preparations, however, would not oxidize DPNH in the absence of added electron carriers.

DISCUSSION

The methods of preparation based on the use of 0·25 M-sucrose (Dounce, 1955) have been successfully applied to the isolation of nuclei from calf thymus by Allfrey *et al.* (1955). This tissue has a high nuclear to cytoplasmic ratio and is relatively free of blood. It is, however, difficult to obtain thymus gland immediately after the death of the animal and Logan *et al.* (1959) have pointed out that these thymus nuclear preparations are liable to contamination with thymocytes.

For our investigation, rat liver was used because further work is proposed on nuclear changes in hepatocarcinogenesis. The nuclear to cytoplasmic ratio is lower than in thymus gland and there is a large quantity of blood in the normal liver. The liver was perfused *in situ* to remove the blood. Five washes and centrifugings were used to remove cell debris. The preparation was completed in 45 min. by using high-speed centrifuging for short times, and was as free from contamination as those prepared with low-speed centrifugings.

Chemical analysis of these preparations gave constant results with a RNA/DNA ratio of 0·97, which is in good agreement with 0·975 obtained by Kay, Smellie, Humphrey & Davidson (1956) for rabbit-embryo liver nuclei in a sucrose medium. However, Naora & Takeda (1954) obtained a ratio of 0·333 for rat-liver nuclei isolated in non-aqueous media (1938) by the Behrens procedure. Kay & Davidson (1955) demonstrated that when liver nuclei isolated by non-aqueous media are treated with sucrose solution, there are losses of protein and RNA. However, the ratio of 0·333 obtained by Naora & Takeda must indicate losses of RNA from the nuclei during non-aqueous isolation, since if the DNA figures obtained in our preparations are adjusted to 100 % recovery and no loss of RNA is assumed, the ratio was 0·45. The findings of Kay & Davidson (1955) may be due to losses of non-nuclear RNA and protein from the heavily contaminated non-aqueous nuclei.

The preparations of rat-liver nuclei described in this paper were found *in vitro* to incorporate [2-^{14}C]glycine into nuclear proteins and [8-^{14}C]-adenine and [6-^{14}C]orotic acid into nuclear RNA. Similar results with rat-liver nuclei and [8-^{14}C]-adenine and [2-^{14}C]phenylalanine have been obtained by Logan *et al.* (1959). Rat-liver nuclei were thus similar in this respect to the preparations from calf-thymus gland. Linear rates of incorporation were obtained with the rat-liver nuclei for periods of up to 190 min. The incorporation rate is

lower in rat-liver nuclei than in calf-thymus nuclei, and there is no change in rates of incorporation when sodium ions in the incubation medium are replaced by potassium ions.

The synthetic reactions described in this paper were inhibited by 2:4-dinitrophenol, azide and anoxia. This would suggest that the energy for these reactions depends on a mechanism such as oxidative phosphorylation. Allfrey, Mirsky & Osawa (1957) came to similar conclusions about thymus nuclei, and Osawa, Allfrey & Mirsky (1957) demonstrated the presence of nuclear adenine nucleotides which became phosphorylated in the presence of oxygen. Naora & Takeda (1954) claim that 45% of the total cell ATP of the rat liver is in the nucleus. However, the nuclei described in this paper did not contain free adenine nucleotides. This may well be a result of the isolation procedure (Keir & Davidson, 1958).

In liver microsomes, incorporation of amino acids is enhanced by the addition of ATP and cell sap (Zamecnik & Keller, 1954), whereas with nuclei these factors are inhibitory. This difference may be caused by structural differences which in the nuclei may result in barriers against the entry of molecules, such as ATP or polynucleotides, which play a role in amino acid incorporation by microsomes (Lipmann, 1958).

The stimulation of nuclear adenosine triphosphatase by 2:4-dinitrophenol and the effects of chlorpromazine support the idea of oxidative phosphorylation. Chlorpromazine is known to act by inhibition of flavoproteins (Dawkins *et al.* 1959, 1960) and the inhibition of the synthetic reactions by this agent suggests that a flavoprotein enzyme is involved in the production of energy for the synthetic reactions. Such a flavoprotein enzyme could be DPNH–cytochrome *c* reductase, which has been shown to be present. The presence of a DPNH–neotetrazolium reductase in the nucleus suggests that the flavoprotein DPNH–cytochrome *c* reductase may be coupled with a quinone like ubiquinone. Martius (1956) has shown that the quinone vitamin K is present in rat-liver nuclei.

It is thus possible to visualize a series of reactions to generate ATP. An electron chain oxidizing DPNH via a flavoprotein enzyme to a quinone could be coupled with a phosphorylation mechanism similar to that in the mitochondrion. This leaves the problem of explaining how the reduced quinone is reoxidized. In theory this could be achieved by an anaerobic mechanism whereby electrons are passed to an acceptor such as unsaturated fatty acids. In view of the inhibitory effects of anoxia in synthetic reactions and the effects of aeration on maintaining the ATP/ADP ratio in thymus nuclei described by Osawa *et al.* (1957), it would seem likely that an oxidative mechanism is involved. However, the fact that DPNH is not oxidized directly by nuclear preparations without the addition of electron acceptors demonstrates that the quinone is not oxidized by atmospheric oxygen. In mitochondria the oxidation of reduced ubiquinone can take place via the cytochrome system (Green, 1959). The results obtained in this paper suggest that cytochrome oxidase is present in the nucleus and hence a similar mechanism of oxidation may exist.

SUMMARY

1. A method for the isolation of rat-liver nuclei in 0·25 M-sucrose has been described, and the nucleic acid and phospholipid content have been determined.

2. The nuclei incorporate ^{32}P, [8-^{14}C]adenine, [6-^{14}C]orotic acid and [2-^{14}C]glycine into their nucleic acids or protein *in vitro*.

3. Anoxia, 2:4-dinitrophenol, azide and chlorpromazine inhibit the incorporation into the protein and nucleic acid.

4. The adenosine-triphosphatase activity of the nuclear preparations has been measured, and the effects of inhibitors have been studied.

5. The presence of cytochrome oxidase, reduced diphosphopyridine nucleotide–cytochrome *c* reductase and reduced diphosphopyridine nucleotide-neotetrazolium reductase have been demonstrated in the nuclear preparations.

6. A possible scheme for the production of adenosine triphosphate in the nucleus is discussed.

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The Reduction of Acetoacetate to β-Hydroxybutyrate in Animal Tissues

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Krebs & Eggleston (1948) reported that minced sheep-heart muscle, when suspended in a saline medium, reduced acetoacetate to β-hydroxybutyrate more rapidly under aerobic than under anaerobic conditions. It is somewhat unexpected that oxygen should promote a reduction and the present investigation was undertaken to throw light on the mechanism by which this effect of oxygen is brought about.

EXPERIMENTAL

Materials

Sodium acetoacetate was prepared as described by Krebs & Eggleston (1945). Stock solutions were stored at −18°. Because of its instability the concentration of the acetoacetate solution used was determined for each experiment.

Sodium DL-β-hydroxybutyrate was obtained from British Drug Houses Ltd. The product was eluted as a single peak when chromatographed on a Celite column (Utter & Swim, 1957) and recoveries of the eluted free acid, based on titration against standard NaOH, were 103% in two successive experiments. Samples of sodium D(−)- and L(+)-β-hydroxybutyrate were kindly given by Dr G. D. Greville (see Lehninger & Greville, 1953).

Adenosine triphosphate (ATP) was obtained from Schwartz Chemical Co., U.S.A. Dicoumarol was the product of Organon Laboratories Ltd.

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Tissue preparations

Homogenates were prepared in a saline medium which consisted of 100 vol. of 0·155 M-KCl, 10 vol. of 0·1 M-potassium phosphate buffer, pH 7·4, and 1 vol. of 0·1 M-$MgCl_2$.

Pig and sheep hearts were obtained from an abattoir. They were placed in ice immediately after removal from the animal and reached the laboratory in 15–20 min. Other tissues were immersed in ice-cooled saline medium immediately after removal from the animal. The tissue was freed from fat and connective tissue, minced in a chilled Fischer mincer (Jouan, Paris) and homogenized in a stainless steel Potter–Elvehjem homogenizer with 6·5 vol. of cold (0°) saline medium.

Incubations were carried out in conical Warburg flasks at 30°. The centre wells of vessels contained 2 N-NaOH and filter paper in aerobic experiments, and a stick of yellow phosphorus in anaerobic experiments. The main compartments contained 3·0 ml. of homogenate (approx. 70 mg. dry wt. of tissue). Substrates were placed in the side arm in the form of a 0·1–0·2 M-solution of the sodium salt and the total volume was made up to 4·0 ml. with saline medium. The vessels were kept in ice during the preparation of the experiment. Unless stated otherwise the substrates were added after 5 min. incubation at 30° and the vessels were incubated for a further 45 min. At the end of the incubation the vessels were cooled in ice and 2·0 ml. of the contents of each vessel was transferred to a tube containing 0·5 ml. of N-HCl (solution A). These tubes were stored at −18°. To the remaining 2·0 ml. in each vessel 0·5 ml. of 25% (w/v) trichloroacetic acid was added and the

precipitated proteins were removed by centrifuging at 0°. The supernatants (solution B) were stored in a refrigerator and used for acetoacetate determinations on the same day.

Chemical methods

Determination of acetoacetate. Unless otherwise stated the method of Walker (1954) was used. The calibration curve was prepared with a solution of acetoacetate standardized by the aniline citrate method of Edson (1935).

Determination of β-hydroxybutyrate. To 1·0 ml. of solution A (see above) in a 10 ml. conical centrifuge tube were added in turn 2·0 ml. of 0·1 N-NaOH, 2·0 ml. of 0·15 N-$Ba(OH)_2$ and 2·0 ml. of 2·5% (w/v) $ZnSO_4,7H_2O$, and the contents of the tube were mixed and centrifuged. A 5·0 ml. portion of the supernatant was measured into a 10 ml. centrifuge tube, and 0·5 ml. of 20% (w/v) $CuSO_4$, $5H_2O$ and 0·5 ml. of 10% (w/v) $Ca(OH)_2$ suspension were added with mixing. The mixture was kept for 15 min. and then centrifuged. The supernatant was transferred to a 28 mm. × 150 mm. test tube, and a drop of conc. H_2SO_4 and a few alundum chips were added. After weighing the tube and contents the solution was boiled for 10 min. to remove acetoacetate, and the original weight was restored by addition of distilled water. β-Hydroxybutyrate was estimated in 3·0 ml. of this solution by the following modification of the method of Greenberg & Lester (1944) and Lester & Greenberg (1948). Each addition of $K_2Cr_2O_7$ was followed by a 15 min. (instead of a 10 min.) refluxing period. After the oxidation steps, the dichromate was reduced with 0·5 ml. of freshly prepared 30% (w/v) $Na_2SO_3,7H_2O$. The solvent used for the 2:4-dinitrophenylhydrazine was N-HCl as it gave a lower reagent blank and more consistent results than 2 N-HCl. The extinction of the phenylhydrazone was measured at 420 mμ.

Isolation of β-hydroxybutyrate. This was carried out according to Utter & Swim (1957) with a Celite column, containing either 5 g. or 50 g. of Celite, the volumes of the fractions being 5 ml. and 50 ml. respectively. β-Hydroxybutyrate was eluted in fractions 22–26. As the elution peak of β-hydroxybutyrate overlaps that of lactate the chromatography had to be repeated several times in order to obtain a sample of β-hydroxybutyrate free from lactate.

Oxygen consumption. The O_2 consumption was measured manometrically at 10 min. intervals, between 10 and 40 min. after adding the contents of the side arm. In calculating the total O_2 consumption during incubation, it was assumed that the rates between 0 and 10 min. were the same as between 10 and 20 min., and that the rates between 40 and 45 min. were the same as between 30 and 40 min. As the O_2 uptake was usually fairly constant, no major errors were introduced by this extrapolation, with the possible exception of some experiments with dinitrophenol concentrations above 0·01 mM where the O_2 uptake fell sharply during the incubation. An error arising from this fall would make values too low.

RESULTS

Metabolism of acetoacetate in homogenates of various tissues

Effect of oxygen. Oxygen increased the rate of acetoacetate removal in the four tissues tested, but sheep heart was the only tissue where oxygen also increased the rate of β-hydroxybutyrate formation from acetoacetate (Table 1). In pigeon heart, pig heart and rat-kidney cortex, the amounts of β-hydroxybutyrate formed aerobically represented 15–40% of the acetoacetate removed, whereas in sheep heart it was 50–75% (Tables 1 and 2). The

Table 1. *Acetoacetate removal and β-hydroxybutyrate formation in various tissue homogenates: effects of oxygen, adenosine triphosphate, ethylenediaminetetra-acetic acid and dinitrophenol*

For general conditions see text. The complete system contained 4 μmoles of ATP and 1 μmole of EDTA in 4 ml. and approx. 40 μmoles of acetoacetate. Dinitrophenol was present in the main compartment from the beginning. The acetoacetate was added from the side arm immediately before placing the vessels in the water bath.

				Aerobic changes		Anaerobic changes	
Tissue	Incubation time (min.)	Modification of incubation mixture	Dinitrophenol (mM)	Acetoacetate removal (μmoles)	β-Hydroxybutyrate formation (μmoles)	Acetoacetate removal (μmoles)	β-Hydroxybutyrate formation (μmoles)
Pigeon heart	60	None	0	12·0	1·6	3·3	2·5
		No ATP	0	12·8	2·2	3·8	3·0
Pigeon heart	50	None	0	8·2	1·8	2·9	2·3
		None	0·20	1·4	0·4	1·7	2·3
		No EDTA	0	5·5	1·7	2·5	2·2
Pig heart	30	None	0	4·9	2·1	3·0	3·4
		None	0·20	0·2	0·3	2·1	2·3
		No EDTA	0	5·4	2·1	2·6	3·2
Rat-kidney cortex	45	None	0	10·0	1·8	4·8	2·4
		None	0·20	1·4	0·4	3·8	2·1
		No EDTA	0	6·5	1·4	1·4	—
Sheep heart	45	None	0	11·6	9·4	4·6	6·3
		None	0·20	0·1	1·3	3·4	3·9
		No EDTA, no ATP	0	13·0	9·1	4·6	5·3
		No EDTA, no ATP	0·20	0·9	1·6	4·1	4·3

Table 2. *Time course of the metabolism of acetoacetate in sheep-heart homogenates at different substrate concentrations*

For experimental conditions see text. The data refer to 4 ml. of homogenate. Different homogenates were used for the aerobic and anaerobic incubation.

		Acetoacetate (9·6 μmoles) added		Acetoacetate (19·2 μmoles) added		Acetoacetate (38·4 μmoles) added	
Gas	Incubation time (min.)	Aceto-acetate removal* (μmoles)	β-Hydroxy-butyrate formation (μmoles)	Aceto-acetate removal (μmoles)	β-Hydroxy-butyrate formation (μmoles)	Aceto-acetate removal (μmoles)	β-Hydroxy-butyrate formation (μmoles)
N_2	10	2·5	2·5	3·0	2·6	0·6	3·3
	20	3·2	3·0	3·0	3·3	1·9	3·6
	40	3·7	3·7	3·3	3·7	3·3	4·8
O_2	10	6·0	3·3	6·5	3·3	7·9	3·4
	20	8·0	4·2	9·6	5·7	11·5	5·6
	40	8·5	4·0	13·9	7·4	15·9	8·7

stimulation of β-hydroxybutyrate formation by oxygen, described by Krebs & Eggleston (1948), is thus peculiar to sheep heart. Anaerobically, β-hydroxybutyrate was the principal product formed from acetoacetate in all four tissues.

Effect of 2:4-dinitrophenol. A striking effect was obtained. In all the tissues examined, 0·20 mM-dinitrophenol strongly inhibited (80–90%) the aerobic acetoacetate removal and β-hydroxybutyrate formation. Anaerobically, there was a very much smaller inhibition (Table 1).

Effects of ethylenediaminetetra-acetic acid and adenosine triphosphate. Omission of ethylene diaminetetra-acetic acid (EDTA) (0·25 mM) lowered the aerobic acetoacetate removal in rat-kidney cortex and pigeon heart but had no appreciable effect on the formation of β-hydroxybutyrate (Table 1). There were no major effects in pig heart and sheep heart. The omission of ATP, where tested, made no appreciable difference.

Because sheep heart was the only tissue in which oxygen stimulated the formation of β-hydroxybutyrate, it was used for most other experiments. EDTA and ATP were omitted in subsequent experiments (except when otherwise stated) because they had no effect in this tissue.

Acetoacetate metabolism in sheep-heart homogenates

Time course. Anaerobically, the removal of acetoacetate and the equivalent formation of β-hydroxybutyrate usually came to a stop after about 20 min. (Table 2), presumably because of lack of hydrogen donors. Aerobically, both acetoacetate removal and β-hydroxybutyrate formation usually progressed throughout an incubation period of 80 min., though the rates gradually decreased.

Effect of substrate concentration. An increase in substrate concentration from 2·4 to 9·6 mM (Table 2) had no appreciable effect on the rate of the anaerobic metabolism and on the initial aerobic rates, but the rate of the aerobic formation of β-hydroxybutyrate fell when the concentration of acetoacetate became low in the course of the incubation (Table 2).

Metabolism of the optical isomerides of β-hydroxybutyrate. The different effects of dinitrophenol on the aerobic and anaerobic metabolism of the ketone bodies suggested that different isomerides of β-hydroxybutyrate might be involved aerobically and anaerobically. It is known (Lehninger & Greville, 1953; McCann, 1957) that acetoacetate can be directly reduced to D(−)-β-hydroxybutyrate, whereas the reduction to the L(+)-form requires a prior conversion of acetoacetate into its coenzyme A ester. As this conversion depends on the supply of succinyl-coenzyme A through the tricarboxylic acid cycle, it would be expected to be accelerated by respiration. Further, the oxidation–reduction potential of the coenzyme A esters of acetoacetate and β-hydroxybutyrate is estimated to be considerably less negative [E° (pH 7·0), −0·238 v] than that of the free acids [E° (pH 7·0), −0·349 v] (Burton, 1957). This implies that the coenzyme A derivatives are more readily reducible by pyridine nucleotides than are the free acids. Because of these considerations, the behaviour of the D- and L-forms was investigated. Sheep-heart homogenates removed D(−)-β-hydroxybutyrate 3–5 times as fast as L(+)-β-hydroxybutyrate (Table 3). About one-third of the D(−)-isomeride, but very little of the L(+)-isomeride, which disappeared was converted into acetoacetate. These observations are similar to those of McCann (1957) on rat-heart sarcosomes.

The presence of 0·20 mM-dinitrophenol completely inhibited the removal of the L-form, but stimulated the rate of oxidation of the D-form to acetoacetate (Table 3).

These findings support McCann's (1957) conclusion that the L-form requires conversion into

the coenzyme A derivative, whereas the D-form can dehydrogenate as such. As the formation of L-β-hydroxybutyryl-coenzyme A requires ATP, it is expected to be inhibited in homogenates by dinitrophenol.

The differences in the behaviour of the L- and D-isomerides were used to examine the question of whether different isomerides accumulate in homogenates of sheep-heart muscles aerobically and anaerobically. The β-hydroxybutyrate was isolated from a suspension, containing 300 μmoles of acetoacetate and 22·5 ml. of homogenate in a total volume of 30 ml., after aerobic or anaerobic incubation at 30° for 60 min. The incubated suspensions were deproteinized by the addition of 2 vol. of 0·15N-barium hydroxide and 2 vol. of 2·5% (w/v) zinc sulphate heptahydrate. The mixture was centrifuged, and the precipitate was washed with water. Supernatant and washings were then combined and evaporated to dryness. β-Hydroxybutyrate was isolated from the residue on a column containing 5 g. of Celite according to Utter & Swim (1957). The chromatography was repeated without titration, β-hydroxybutyrate being extracted from the chloroform phase with an equivalent amount of sodium hydroxide solution. The aqueous phase was evaporated to dryness, and the residue was dissolved in a small quantity of water. The concentration of β-hydroxybutyrate in this solution was determined according to Greenberg & Lester (1944).

The material isolated from the aerobic and anaerobic incubations behaved identically when added to respiring sheep-heart homogenate: the rate of removal was of the same order as that of D(−)-β-hydroxybutyrate; it was increased by 0·2 mM-dinitrophenol, and the yield of acetoacetate in the presence of dinitrophenol was almost 100%. These results indicate that the D(−)-isomeride was formed both aerobically and anaerobically.

Measurement of the optical rotation of the β-hydroxybutyrate formed aerobically and anerobically. The above conclusion was confirmed by the following experiment. Latapie-minced sheep heart (100 g.) was homogenized with 650 ml. of ice-cold saline medium in a Waring Blendor and the homogenate was diluted to 1000 ml. with saline medium. Equal portions of the homogenate (500 ml.) were placed in two 1 l. flasks, immersed in a bath at 30°, nitrogen being bubbled through one flask and oxygen through the other. After 5 min., acetoacetate (5 mM) was added to each flask and the incubation was continued for 2 hr. At the end of the incubation, 125 ml. of 25% (w/v) trichloroacetic acid was added to each flask and the precipitated proteins were removed by centrifuging. The supernatants were evaporated to dryness, and β-hydroxybutyrate was isolated from the residue by chromatography on a column containing 50 g. of Celite (Utter & Swim, 1957). The β-hydroxybutyrate fraction was rechromatographed three times to remove traces of other organic acids, principally lactate (Krebs & Eggleston, 1945), which might interfere with optical rotation measurements. The procedure for the final chromatographic separation was similar to that described in the preceding section. The optical rotations observed were as follows. β-Hydroxybutyrate formed aerobically: $[\alpha]_D^{19} - 14{\cdot}5° \pm 1{\cdot}0$ in water (*c*, 8·35); β-hydroxybutyrate formed anaerobically: $[\alpha]_D^{19} - 15{\cdot}1° \pm 1{\cdot}5$ in water (*c*, 2·65). These values are close to the value ($[\alpha]_D^{19} - 14{\cdot}4°$) reported by Lehninger & Greville (1953) for pure sodium D(−)-β-hydroxybutyrate. Thus all the available evidence demonstrates that the product of both the aerobic and anaerobic reduction of acetoacetate is predominantly if not exclusively D(−)-β-hydroxybutyrate.

Effect of dinitrophenol concentration on acetoacetate metabolism (with acetoacetate as only added substrate). When acetoacetate was the sole substrate, the highest increases in the oxygen uptake (up to threefold) were usually obtained with 0·01 mM-dinitrophenol (Tables 4–9). Higher concentrations gave submaximal increases in most experiments and even inhibitions in some. The rates of oxygen uptake were constant without dinitrophenol or with concentrations up to 0·01 mM. At the higher concentrations, the rates fell during

Table 3. *Metabolism of* D(−)- *and* L(+)-β-*hydroxybutyrate in sheep-heart homogenates: effect of dinitrophenol*

For experimental conditions see text. Incubation time was 45 min.

	β-Hydroxybutyrate		No dinitrophenol added		0·2 mM-Dinitrophenol	
Expt. no.	Isomer used	Amount added (μmoles)	β-Hydroxybutyrate removal (μmoles)	Acetoacetate formation (μmoles)	β-Hydroxybutyrate removal (μmoles)	Acetoacetate formation (μmoles)
1	DL	18·8	6·4	2·0	8·3	8·1
	L(+)	8·9	1·7	0·4	0·2	0·4
2	DL	18·8	6·8	1·6	8·1	9·4
	D(−)	8·9	4·5	1·5	8·0	8·3
	L(+)	8·9	1·0	Nil	Nil	Nil

Table 4. *Effect of dinitrophenol concentration on acetoacetate metabolism in sheep-heart homogenates*

For conditions see text. The data refer to 4 ml. of homogenate to which about 40 μmoles of acetoacetate was added. In Expt. 2, the final dilution of the tissue was 1:8, against 1:10 in the usual procedure. The uncoupling agent was added either from the side arm at the same time as the substrate (procedure A) or to the cup contents before incubation (procedure B) as indicated below.

Expt. no.	Dinitrophenol concn. (mM)	Procedure	Gas	Oxygen uptake (μmoles)	Aceto-acetate removal (μmoles)	β-Hydroxy-butyrate formation (μmoles)
1	Nil	A	O_2	25·0	12·2	5·8
	0·005			40·2	13·0	4·7
	0·01			50·0	14·6	3·4
	0·02			25·1	2·1	0·5
	0·04			21·9	Nil	Nil
	0·08			15·6	Nil	Nil
	0·005	B		38·6	11·9	4·5
	0·01			48·7	13·5	—
	0·02			22·6	1·5	—
	0·04			16·4	Nil	—
	0·08			14·5	Nil	—
2	Nil	A	O_2	36·3	15·7	7·9
	0·005			51·8	15·7	6·0
	0·01			63·8	16·2	3·4
	0·02			84·0	17·0	3·0
	0·04			60·8	2·8	0·6
	0·08			37·1	1·3	—
	0·16			32·3	1·3	—
	Nil		N_2	—	6·2	4·9
	0·01			—	6·2	4·9
	0·16			—	4·1	3·8

Table 5. *Comparison of the effect of dinitrophenol and dicoumarol on acetoacetate metabolism in sheep-heart homogenates*

The data refer to 4 ml. of homogenate with about 40 μmoles of acetoacetate. The 'uncoupling agent' was added from the side arm at the same time as the substrate.

Expt. no.	Uncoupling agent	Concn. (mM)	Gas	Oxygen uptake (μmoles)	Aceto-acetate removal (μmoles)	β-Hydroxy-butyrate formation (μmoles)
1	Nil		O_2	20·4	14·8	10·0
	Dinitrophenol	0·01		54·2	15·9	4·2
	Dicoumarol	0·01		39·3	13·9	6·6
	Nil		N_2	—	2·7	3·7
	Dinitrophenol	0·01		—	2·0	3·8
	Dicoumarol	0·01		—	2·3	3·8
2	Nil		O_2	26·6	15·7	7·2
	Dinitrophenol	0·01		58·6	16·8	1·7
	Dinitrophenol	0·10		11·9	0·7	Nil
	Dicoumarol	0·01		55·0	15·7	2·2
	Dicoumarol	0·10		10·0	Nil	Nil

the incubation, as had been noted before under other conditions by Tyler (1950) and Simon (1953).

The amounts of acetoacetate removed were not much affected by 0·01 mM-DNP, whereas the ratio of β-hydroxybutyrate formed to acetoacetate removed was decreased from 0·5–0·75 to 0·1–0·35 (Tables 4–9). In contrast, dinitrophenol had no major effects on the anaerobic acetoacetate metabolism.

These effects of dinitrophenol are similar to those on acetate oxidation in rat-kidney homogenates observed by Aisenberg & Potter (1955), who attributed the effect to an inhibition of acetate activation by dinitrophenol. An inhibition by it of the oxidation of acetoacetate and fatty acids in mitochondria has already been reported by Cross, Taggart, Covo & Green (1949) and Witter, Newcomb & Stotz (1953).

Effects of dicoumarol. Dicoumarol, another agent uncoupling oxidative phosphorylation (Martius & Nitz-Litzow, 1953), decreased the aerobic β-hydroxybutyrate formation at a concentration of 0·01 mM to about the same degree as dinitrophenol (Table 5) without appreciably influencing the total

amount of acetoacetate removed. The decreased aerobic β-hydroxybutyrate formation was accompanied by an increased oxygen uptake. 0·1 mM-Dicoumarol, like dinitrophenol, strongly inhibited the aerobic acetoacetate removal.

Effect of adenosine triphosphate. Since uncoupling agents are known to cause a decrease in the ATP concentration, it might be thought that addition of ATP might counteract the effects of dinitrophenol. This did not prove to be the case. 2·5 mM-ATP stimulated aerobic acetoacetate removal and β-hydroxybutyrate formation by 20–35 % without altering the ratio of β-hydroxybutyrate formed to acetoacetate removed (Table 6). This concentration of ATP had no effect on the anaerobic reduction of acetoacetate. In the presence of dinitrophenol (0·05 or 0·2 mM) ATP inhibited the oxygen uptake by about 25 %.

Effect of α-oxoglutarate and dinitrophenol. Dinitrophenol trebled the oxygen consumption in the presence of α-oxoglutarate and caused a still greater increase (up to fivefold) of oxygen uptake when both α-oxoglutarate and acetoacetate were added (Table 7). α-Oxoglutarate stimulated the

Table 6. *Effect of adenosine triphosphate on acetoacetate metabolism in sheep-heart homogenates*

The data refer to 4 ml. of homogenate with about 40 μmoles of acetoacetate. The substrate (acetoacetate) and dinitrophenol were added from the side arm.

			Aerobic changes				Anaerobic changes	
Expt. no.	ATP concn. (mM)	Dinitrophenol concn. (mM)	Oxygen uptake (μmoles)	Acetoacetate removal (μmoles)	β-Hydroxybutyrate formation (μmoles)	Ratio β-hydroxybutyrate formation/acetoacetate removal	Acetoacetate removal (μmoles)	β-Hydroxybutyrate formation (μmoles)
1	0	0	18·1	13·4	8·6	0·64	3·2	3·3
	2·5	0	19·7	16·2	10·4	0·64	3·2	3·4
	0	0·05	15·6	1·3	0·8	—	3·9	3·8
	2·5	0·05	12·0	0·2	Nil	—	3·9	3·3
2	0	0	16·3	14·7	10·9	0·74	—	—
	2·5	0	15·4	19·8	15·3	0·77	—	—
	0	0·20	32·3	2·0	0·8	—	—	—
	2·5	0·20	23·7	1·8	—	—	—	—

Table 7. *Effect of α-oxoglutarate on acetoacetate metabolism in sheep-heart homogenates*

The data refer to 4 ml. with about 40 μmoles of acetoacetate or 40 μmoles of α-oxoglutarate, or both; dinitrophenol was added from the side arm at the same time as the substrate.

Expt. no.	Substrate	Dinitrophenol concn. (mM)	Gas	Oxygen uptake (μmoles)	Acetoacetate removal (μmoles)	β-Hydroxybutyrate formation (μmoles)	Ratio β-hydroxybutyrate formation/acetoacetate removal
1	Acetoacetate	0	O_2	17·4	16·4	10·7	0·65
		0·01		61·2	16·4	5·4	0·33
		0·05		34·9	1·9	1·0	—
		0·20		32·2	1·9	0·7	—
	α-Oxoglutarate	0		20·5	—	—	—
		0·01		60·8	—	—	—
		0·05		68·0	—	—	—
		0·20		72·3	—	—	—
	Acetoacetate + α-oxoglutarate	0		23·0	21·0	13·5	0·64
		0·01		69·0	24·4	8·6	0·35
		0·05		106·5	21·0	6·9	0·33
		0·20		93·3	21·2	10·0	0·47
2	Acetoacetate	0	O_2	16·3	14·7	10·9	0·74
		0·01		58·8	15·7	5·2	0·33
		0·20		32·0	2·0	0·8	—
	Acetoacetate + α-oxoglutarate	0		19·7	17·7	13·3	0·75
		0·01		70·7	22·7	8·1	0·36
		0·20		99·0	13·9	3·3	0·24
	Acetoacetate	0	N_2	—	5·2	6·6	—
	Acetoacetate + α-oxoglutarate	0		—	14·5	15·3	—
		0·01		—	13·2	12·5	—
		0·20		—	8·2	11·3	—

Table 8. *Effect of fumarate on acetoacetate metabolism in sheep-heart homogenates*

The data refer to 4 ml. of homogenate with about 40 μmoles of acetoacetate or 40 μmoles of fumarate, or both. Dinitrophenol was added from the side arm at the same time as the substrate.

Expt. no.	Substrate	Dinitrophenol concn. (mM)	Gas	Oxygen uptake (μmoles)	Acetoacetate removal (μmoles)	β-Hydroxybutyrate formation (μmoles)	Ratio β-hydroxybutyrate formation/acetoacetate removal
1	Acetoacetate	0	O_2	23·5	12·2	7·2	0·59
		0·01		52·0	12·9	3·9	0·30
		0·05		23·0	1·1	0·7	—
		0·20		18·6	0·4	0·3	—
	Fumarate	0		25·4	—	—	—
		0·01		38·8	—	—	—
		0·05		35·0	—	—	—
		0·20		25·0	—	—	—
	Acetoacetate + fumarate	0		31·9	16·3	9·6	0·59
		0·01		58·3	18·6	5·6	0·30
		0·05		40·7	2·2	0·7	—
		0·20		33·9	1·1	0·4	—
2	Acetoacetate	0	O_2	23·9	14·5	9·2	0·63
		0·01		57·8	16·6	4·9	0·30
		0·20		15·6	0·9	—	—
	Acetoacetate + fumarate	0		30·9	20·5	12·2	0·60
		0·01		62·2	22·8	7·7	0·34
		0·20		25·6	0	—	—
	Acetoacetate	0	N_2	—	4·3	3·6	—
		0·01		—	4·7	5·3	—
		0·20		—	3·8	5·0	—
	Acetoacetate + fumarate	0		—	21·8	20·2	—
		0·01		—	22·5	22·9	—
		0·20		—	14·5	13·0	—

Table 9. *Effect of pyruvate and lactate on acetoacetate metabolism in sheep-heart homogenates*

Data refer to 4 ml. of homogenate with about 40 μmoles of acetoacetate alone or together with 40 μmoles of pyruvate or L(+)-lactate. In Expts. 1 and 2 dinitrophenol was added from the side arm together with the substrate; in Expts. 3 and 4 it was placed in the main compartment.

			Oxygen			Nitrogen	
Expt. no.	Substrate	Dinitrophenol concn. (mM)	Oxygen uptake (μmoles)	Acetoacetate removal (μmoles)	β-Hydroxybutyrate formation (μmoles)	Acetoacetate removal (μmoles)	β-Hydroxybutyrate formation (μmoles)
1	Acetoacetate	0	22·1	14·4	7·9	4·2	3·7
		0·01	59·3	13·3	2·6	3·7	3·9
		0·05	16·1	1·1	—	4·6	3·7
	Acetoacetate + pyruvate	0	25·7	12·1	8·9	9·6	8·9
		0·01	62·5	9·8	4·1	7·8	7·5
		0·05	111·5	5·9	2·7	6·4	6·3
2	Nil	0	21·3	—	—	—	—
	Acetoacetate	0	26·0	15·6	11·7	6·7	5·2
	Acetoacetate + pyruvate	0	30·3	12·9	9·7	9·7	10·6
		0·20	83·0	5·9	3·5	4·0	6·4
3	Acetoacetate	0	18·2	15·4	9·2	2·5	3·3
		0·20	11·6	1·6	1·2	2·5	3·5
	Acetoacetate + pyruvate	0	18·8	14·0	10·8	11·2	9·2
		0·20	65·5	4·2	3·6	6·0	4·6
	Acetoacetate + lactate	0	19·8	13·0	8·9	7·4	4·0
		0·20	12·5	−2·2	0·7	4·9	3·5
4	Nil	0	16·5	—	—	—	—
	Acetoacetate	0	20·1	13·3	7·9	3·9	5·2
		0·20	17·7	2·4	1·2	3·3	4·1
	Acetoacetate + lactate	0	23·6	13·1	7·4	6·7	5·4
		0·20	23·2	2·3	1·0	4·4	4·0

Table 10. *Effect of succinate on acetoacetate metabolism in sheep-heart homogenate*

The data refer to 4 ml. of homogenate with about 40 μmoles of acetoacetate. Succinate was 80 μmoles, unless otherwise stated.

Expt. no.	Substrate	Temperature	Period of incubation (min.)	Oxygen			Nitrogen	
				Oxygen uptake (μmoles)	Aceto-acetate removal (μmoles)	β-Hydroxy-butyrate formation (μmoles)	Aceto-acetate removal (μmoles)	β-Hydroxy-butyrate formation (μmoles)
1	None	30°	40	16·5	—	—	—	—
	Acetoacetate		40	24·2	9·3	7·0	8·8	4·9
	Acetoacetate + succinate		40	47·2	15·0	12·1	7·3	4·9
2	Acetoacetate	25	20	8·8	3·8	1·7		
	Acetoacetate		40	18·7	5·1	3·8		
	Acetoacetate		60	25·6	4·8	3·8		
	Acetoacetate + succinate		20	21·4	3·3	2·9		
	Acetoacetate + succinate		40	37·8	7·0	5·7		
	Acetoacetate + succinate		60	45·5	8·1	6·6		
3	Acetoacetate	30	45	33·8	9·7	3·4		
	Acetoacetate + succinate		45	65·8	13·0	6·0		
4	None	30	0	0	—	0·6		
	None		45	19·8	—	0·5		
	Acetoacetate		45	34·3	9·5	4·0		
	Acetoacetate + succinate (2 μmoles)		45	34·5	8·8	4·7		
	Acetoacetate + succinate (20 μmoles)		45	38·0	11·1	6·2		
	Acetoacetate + succinate (80 μmoles)		45	60·5	11·8	9·1		

aerobic removal of acetoacetate (see Krebs & Eggleston, 1945) and almost completely reversed the inhibition of the aerobic removal of acetoacetate by dinitrophenol. The aerobic formation of β-hydroxybutyrate was only partially restored by α-oxoglutarate, so that the ratio of β-hydroxybutyrate formed to acetoacetate removed was still decreased in the presence of dinitrophenol and α-oxoglutarate. Anaerobically, α-oxoglutarate substantially increased the removal of acetoacetate (Krebs & Eggleston, 1945), and this reaction was slightly inhibited by dinitrophenol.

Effect of fumarate and dinitrophenol. The action of fumarate differed in several respects from that of α-oxoglutarate (Tables 7 and 8). The stimulation of oxygen consumption by dinitrophenol was smaller with fumarate than with α-oxoglutarate. The higher concentrations of dinitrophenol (0·05 and 0·2 mM), which did not abolish the stimulation of oxygen consumption produced by 0·01 mM in the presence of α-oxoglutarate, did so in the presence of fumarate, and of acetoacetate and fumarate. In contrast with α-oxoglutarate, fumarate failed to restore aerobically the inhibitory effects of 0·2mM-dinitrophenol on the acetoacetate removal. Anaerobically, fumarate greatly accelerated the removal of acetoacetate and the formation of β-hydroxybutyrate, as was already shown by Krebs & Eggleston (1945). This anaerobic reaction was inhibited (30%) by 0·2 mM, but was not affected by 0·01 mM-dinitrophenol.

Effects of pyruvate, lactate and dinitrophenol. Dinitrophenol caused very large increases in the oxygen uptake when both pyruvate and acetoacetate were present, but not in the presence of L(+)-lactate and acetoacetate (Table 9). Unlike α-oxoglutarate, pyruvate did not fully abolish the inhibition by dinitrophenol of the aerobic removal of acetoacetate, though it decreased the inhibition slightly at 0·2 mM-dinitrophenol. Lactate had no effects on the aerobic acetoacetate removal or β-hydroxybutyrate formation.

Anaerobically, pyruvate and lactate increased both acetoacetate removal and β-hydroxybutyrate formation. Lactate increased the former but not the latter. 0·20 mM-Dinitrophenol largely abolished the accelerating effects of pyruvate and lactate.

Effect of succinate. α-Oxoglutarate, fumarate and pyruvate raised the rate of reduction of acetoacetate aerobically and anaerobically and, in the presence of these substrates, the anaerobic rate was always greater than the aerobic one. In the experiments reported so far, the only material in which the reduction was more rapid aerobically than anaerobically was the suspension unsupplemented by substrates. In further experiments many other substrates were tested, and succinate was the only one which augmented the

Table 11. *Relationships between amount of oxygen consumed and acetoacetate oxidized in sheep heart*

The data are calculated from other tables, as indicated. 'Acetoacetate oxidized' is taken to be the difference between acetoacetate removal and β-hydroxybutyrate formation. The ratio given in the last vertical column is calculated by considering two molecules of β-hydroxybutyrate equivalent to one molecule of O_2. DNP, dinitrophenol.

Table no.	Expt. no.	Substances added	Oxygen uptake (μmoles)	Aceto-acetate oxidized (μmoles)	Oxygen used / Acetoacetate oxidized	β-Hydroxy-butyrate formation (μmoles)	O_2 equivalents used / Acetoacetate oxidized
4	1	Acetoacetate	25·0	6·4	3·9	5·8	4·4
		Acetoacetate + DNP (0·01 mM)	50·0	11·2	4·5	3·4	4·6
4	2	Acetoacetate	36·3	7·8	4·7	7·9	5·1
5	1	Acetoacetate	20·4	4·8	4·3	10·0	5·3
5	2	Acetoacetate	26·6	8·5	3·1	7·2	3·6
7	1	Acetoacetate	17·4	5·7	3·1	10·7	4·0
		Acetoacetate + α-oxoglutarate	23·0	7·5	3·1	13·5	4·0
		Acetoacetate + α-oxoglutarate + DNP (0·01 mM)	69·0	15·8	4·4	8·6	4·6
7	2	Acetoacetate	16·3	3·8	4·4	10·9	5·5
		Acetoacetate + α-oxoglutarate	19·7	4·4	4·4	13·3	6·0
		Acetoacetate + α-oxoglutarate + DNP (0·01 mM)	70·7	14·6	4·8	8·1	5·1
8	1	Acetoacetate	23·5	5·0	4·7	7·2	5·4
		Acetoacetate + fumarate	31·9	6·7	4·8	9·6	5·4
8	2	Acetoacetate	23·9	5·3	4·5	9·2	5·4
		Acetoacetate + fumarate	30·9	8·3	3·7	12·2	4·5
9	1	Acetoacetate	22·1	6·5	3·4	7·9	4·0
		Acetoacetate + pyruvate	25·7	3·2	8·0	8·9	9·6
		Acetoacetate + pyruvate + DNP (0·01 mM)	62·5	5·7	11·0	4·1	11·2
9	2	Acetoacetate	26·0	3·9	6·7	11·7	8·2
		Acetoacetate + pyruvate	30·3	3·2	9·5	9·7	11·0
		Acetoacetate + pyruvate + DNP (0·02 mM)	83·0	2·4	34·5	3·5	35·2
9	4	Acetoacetate	20·1	5·4	3·7	7·9	4·4
		Acetoacetate + lactate	23·6	5·7	4·1	7·4	4·8
10	4	Acetoacetate	34·3	5·5	6·2	4·0	6·6
		Acetoacetate + succinate (80 μmoles)	60·5	2·7	22·4	9·1	24·0

difference between the aerobic and anaerobic reduction rates. Anaerobically, succinate had no effect but, aerobically, increases of up to 100% occurred (Table 10).

DISCUSSION

The experiments show that the fate of acetoacetate in sheep-heart preparations is readily influenced by environmental conditions, the main factors determining the fate being the presence or absence of oxygen, the concentration of other metabolites such as α-oxoglutarate, fumarate, pyruvate or succinate, and the addition of agents uncoupling oxidative phosphorylation. Many of the observations can be accounted for on the basis of the established enzymic reactions of acetoacetate and the known effects of the uncoupling agents.

Stimulation of β-hydroxybutyrate formation by oxidizable substrates. The acceleration by α-oxoglutarate, fumarate and pyruvate of the anaerobic formation of β-hydroxybutyrate may be ascribed to coupled oxidation–reduction, in which the oxidative process maintains a supply of reduced diphosphopyridine nucleotide (DPN) (see Green, Needham & Dewan, 1937; Krebs & Eggleston, 1945). Such reactions can also take place aerobically, but a simple coupled oxidation–reduction cannot explain the ready aerobic reduction of acetoacetate in the presence of succinate, as the oxidation–reduction potential of the succinate–fumarate couple is more than 300 mV more positive than that of the acetoacetate–β-hydroxybutyrate couple. This accounts for the fact that succinate does not interact with acetoacetate under anaerobic conditions.

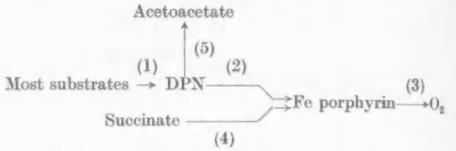
Any explanation of the stimulating effect of succinate on the reduction of acetoacetate must be based on the fact that this reduction requires reduced DPN. Thus it follows that succinate in-

creases the supply of reduced DPN. It is already known from the observations of Chance & Williams (1955*a*, *b*), Chance & Hollunger (1960), Klingenberg, Slenczka & Ritt (1959) and Birt & Bartley (1960) that the level of reduced DPN in mitochondrial suspensions rises when succinate is added. Chance & Hollunger assume that a reduction of DPN by succinate is achieved by what amounts to a reversal of oxidative phosphorylation. They suggest 'that high-energy intermediates formed in the cytochrome portions of the chain by succinate oxidation can be used to reduce DPN' (see also Davies & Krebs, 1952; Krebs & Kornberg, 1957). An alternative explanation is the assumption that in the presence of succinate the transfer of hydrogen from reduced DPN to molecular oxygen is blocked at the iron porphyrin stage, owing to the rapid dehydrogenation of succinate. It is known that hydrogen transport from succinate and from reduced DPN to oxygen shares a common pathway from the iron porphyrin level onwards. Thus the electrons from succinate must compete with those from reduced DPN for the same carrier, and the great increase in the oxygen consumption occurring on addition of succinate (Table 11), as well as the rapid conversion of succinate into fumarate (Krebs, 1960), indicate that the electrons from succinate win in this competition. More reduced DPN therefore becomes available for the conversion of acetoacetate into β-hydroxybutyrate, the supply of reduced DPN being maintained by the dehydrogenation of the products of oxidation of succinate such as malate, pyruvate, *iso*citrate and α-oxoglutarate. The situation is illustrated by Scheme 1.

To sum up it is suggested that a reduction of acetoacetate occurs when the transfer of electrons to DPN [reaction (1)] is more rapid than the transfer of electrons from DPN towards oxygen [reactions (2) and (3)]. Succinate accelerates the reduction of acetoacetate because reaction (4) is more rapid than (2). This causes a competitive inhibition of the transfer of electrons from DPN to oxygen. At the same time, on account of its rapid oxidation, succinate supplies hydrogen donors for DPN.

The accelerating effect of oxygen on the reduction of acetoacetate in sheep-heart homogenates unsupplemented by other substrates is presumably due to the same set of circumstances.

Effects of dinitrophenol. Particularly large increases in the rate of oxygen consumption were caused by dinitrophenol when α-oxoglutarate and acetoacetate, or pyruvate and acetoacetate were present, and with these substrates the higher concentrations of dinitrophenol (0·2 mM) did not appreciably decrease the stimulation caused by



Scheme 1. Pathways of electron transport.

lower concentrations, as they did when acetoacetate alone or acetoacetate plus fumarate were added substrates. In other words the effects of dinitrophenol on the oxygen uptake depend on the nature of the substrates present (Tyler, 1950; Krebs, 1959).

A stimulation of respiration by dinitrophenol is due to increased concentrations of adenosine diphosphate (ADP) arising from the uncoupling of oxidative phosphorylation (Lardy & Wellman, 1952; Aldridge, 1957), and the inhibition of respiration by the higher concentration of dinitrophenol is probably due to loss of ADP, owing to the combined action of adenylate kinase and adenosine triphosphatase. The former causes the formation of adenosine monophosphate (AMP), and, once formed, this is not readily rephosphorylated owing to the low concentration of ATP. In the presence of α-oxoglutarate some ADP can be prevented from entering the adenylate-kinase reaction by conversion into ATP through the dinitrophenol-insensitive α-oxoglutarate-dehydrogenase system (see Hunter & Hixon, 1949). Hence α-oxoglutarate, unlike other substrates of respiration, is expected to prevent, or at least delay, the loss of ADP, a rate-limiting component of respiration.

When dinitrophenol increased the oxygen uptake it also decreased the aerobic β-hydroxybutyrate formation, whereas the aerobic removal of acetoacetate was not affected, and even rose in some cases. The ratio of β-hydroxybutyrate formation to acetoacetate removal therefore fell on addition of dinitrophenol (e.g. Tables 4, 5 and 7–9). This is expected on the basis of Scheme 1. The rate of reaction (5) depends on whether (1) is faster than (2) and (3). Dinitrophenol raises the rate of (2) and (3), and is therefore liable to reduce the rate of (5).

Higher concentration of dinitrophenol (0·2 mM) strongly inhibited the oxidation of acetoacetate when this was the only added substrate or when added together with fumarate or lactate, and this inhibition was much greater than the inhibition of respiration. The oxidation of acetoacetate is known to be mainly initiated by the coenzyme A-transferase reaction (see Stern & Ochoa, 1951; Green, Goldman, Mii & Beinert, 1953; Stern, Coon, del Campillo & Schneider, 1956):

$$\text{succinyl-coenzyme A (CoA)} + \text{acetoacetate} \rightarrow \text{succinate} + \text{acetoacetyl-CoA} \qquad (6)$$

As two molecules of succinyl-coenzyme A are produced in the course of the complete oxidation of one molecule of acetoacetate, there is no obvious reason why dinitrophenol should inhibit acetoacetate oxidation (if its only primary effect is an interference with the synthesis of energy-rich phosphate bonds). However, an explanation for the dinitrophenol effect can be offered on the basis of the fact that succinyl-coenzyme A takes part in a second series of reactions involving ATP, the overall effect of which is:

$$\text{succinyl-CoA} + \text{ADP} + \text{phosphate} \rightarrow \text{succinate} + \text{ATP} + \text{CoA}. \quad (7)$$

Since dinitrophenol causes a fall in the concentration of ATP and a rise in that of ADP, reaction (7) may be expected to be accelerated by it. In consequence succinyl-coenzyme A would react according to (7) rather than to (6). This could account for the observed inhibition of acetoacetate removal. The fact that α-oxoglutarate counteracts the inhibitory effect of dinitrophenol is explained by the ready formation of succinyl-coenzyme A from α-oxoglutarate.

Relationship between oxygen uptake and acetoacetate oxidation. The amounts of acetoacetate which disappeared but were not converted into β-hydroxybutyrate must have undergone complete oxidation, since no appreciable quantities of intermediates accumulated. Table 11 indicates that in many experiments in which the amounts of acetoacetate oxidized (i.e. the difference between acetoacetate removed and β-hydroxybutyrate formed) and the oxygen consumed were measured simultaneously, the ratio of oxygen used to acetoacetate oxidized was about 4. Since the complete oxidation of one molecule of acetoacetate requires 4 molecules of oxygen, it follows that acetoacetate served as a chief substrate for respiration. Since β-hydroxybutyrate acted in addition to oxygen as a terminal hydrogen acceptor in these experiments the ratio given in the last vertical column of Table 11 is a more accurate value of the relationship between the oxidative reactions and acetoacetate degradation. 'O_2 equivalents' in this column represent the sum of oxygen used and β-hydroxybutyrate formed, two molecules of the latter being taken as equivalent to one of oxygen.

Since addition of acetoacetate did not cause a large increase in oxygen consumption it must have suppressed the utilization of endogenous substrates.

Substrate competition. Acetoacetate was not only the preferred substrate of respiration when added as sole substrate, but also when the respiration was accelerated by 0·005 or 0·01 mM-dinitrophenol (e.g. Tables 4, 5 and 7–9). But at higher concentrations of dinitrophenol (0·08 to 0·2 mM) acetoacetate no longer serves as the main substrate (e.g. Tables 4, 7, 9). This can be explained by the fact that acetoacetate depends on ATP for its competition with other substrates. As already mentioned, dinitrophenol interferes with conversion of acetoacetate into acetoacetyl-coenzyme A. On the other hand the initiation of the oxidation of many other substrates such as glucose or acetate is dependent on the ATP concentration.

When the rate of acetoacetate oxidation is accelerated by α-oxoglutarate (Table 7) or by fumarate (Table 8) the ratio of oxygen equivalents used to acetoacetate oxidation remained of the same order (Table 11), which indicates that acetoacetate was still the preferred substrate when α-oxoglutarate or fumarate is present. However, with pyruvate there was a considerable decrease in oxidation of acetoacetate, the ratio of oxygen equivalents used to acetoacetate oxidized reaching values of 11. This means that only 36 % of the oxygen uptake was accounted for by oxidation of acetoacetate. Succinate suppressed the oxidation of acetoacetate almost completely.

SUMMARY

1. Acetoacetate, when added aerobically to sheep-heart-muscle homogenates, is partly oxidized to carbon dioxide and water, and partly reduced to D(−)-β-hydroxybutyrate. The ratio of oxygen equivalents used to acetoacetate oxidized is near 4. This suggests that acetoacetate is oxidized in preference to endogenous substrates.

2. In sheep heart, but not in the hearts of rats or pigeons and various other tissues tested, more β-hydroxybutyrate is formed aerobically than anaerobically when acetoacetate is the sole added substrate.

3. α-Oxoglutarate, fumarate and pyruvate accelerate both the anaerobic or aerobic conversion of acetoacetate into β-hydroxybutyrate. This can be explained by coupled oxidation–reduction mediated by diphosphopyridine nucleotide (DPN).

4. Succinate increases the rate of the aerobic reduction of acetoacetate but not of the anaerobic reduction. This is discussed and it is suggested that succinate, on account of its ready dehydrogenation, blocks the transfer of electrons from reduced DPN to the cytochrome system and thereby increases the supply of reduced DPN available for the reduction of acetoacetate.

5. The action of dinitrophenol on acetoacetate metabolism varied with the presence of other substrates. 0·01 mM-Dinitrophenol caused large increases in the oxygen uptake and acetoacetate oxidation, especially when α-oxoglutarate and pyruvate were also added. At 0·2 mM, it inhibited

the oxygen uptake and acetoacetate oxidation when added alone or with fumarate, but not in the presence of α-oxoglutarate. The reasons for these differences are discussed. Anaerobically, relatively slight inhibitions of the reduction of acetoacetate were observed.

6. The oxidation of L(+)-β-hydroxybutyrate is inhibited by dinitrophenol, whereas that of the D(−)-form is not. This is related to the fact that only the L(+)-form requires conversion into the coenzyme A derivative. The differences in the behaviour of the L- and D-forms towards dinitrophenol were used to examine the configuration of the β-hydroxybutyrate formed in sheep-heart muscle aerobically and anaerobically. In both cases the D-form only was found.

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Studies on Glucosaminidase

2. SUBSTRATES FOR *N*-ACETYL-β-GLUCOSAMINIDASE*

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Methods available for the estimation of the activity of *N*-acetyl-β-glucosaminidase have been limited by a lack of suitable substrates. Those with an alkyl *N*-acetyl-β-glucosaminide as substrate (Neuberger & Pitt Rivers, 1939; Kuhn & Tiedemann, 1954) depend on the relatively non-specific estimation of reducing sugar liberated. Phenyl (Helferich & Iloff, 1933) and *p*-nitrophenyl (Westphal & Schmidt, 1952) *N*-acetyl-β-glucosaminide were used as substrates by Watanabe (1936), Neuberger & Pitt Rivers (1939) and Pugh, Leaback & Walker (1957*b*), and by Conchie, Findlay & Levvy (1959) respectively, in methods depending on the colorimetric or spectrophoto-

* Part 1: Pugh, Leaback & Walker (1957*b*).

metric estimation of the liberated phenol. The development of a general method for the synthesis of alkyl and aryl *N*-acetyl-β-glucosaminides (Leaback & Walker, 1957) has extended the range of substrates available. This paper describes the kinetics of hydrolysis of an alkyl and four aryl *N*-acetyl-β-glucosaminides by enzymes from two sources. The suitability of these compounds as substrates for the estimation of *N*-acetyl-β-glucosaminidase is compared.

METHODS AND MATERIALS

Preparation of substrates. Methyl, ethyl, phenyl, *o*- and *p*-nitrophenyl and *p*-acetylphenyl *N*-acetyl-β-glucosaminides were prepared as described by Leaback & Walker (1957). Approximate solubilities of the aryl glycosides (mM) in water at 20–22° are: phenyl, 40; *o*-nitrophenyl and *p*-acetylphenyl, 23; *p*-nitrophenyl, 15.

Buffers. Citrate buffers (0·5 M): these were prepared from stock solutions of sodium citrate and citric acid. In order to obtain the desired final pH in reaction mixtures 0·05 M with respect to citrate, allowance was made for the rise of approx. 0·2 pH unit which takes place on dilution (Kolthoff & Bosch, 1927). Pyrophosphate buffer, pH 9·2: sodium pyrophosphate (10 g.), semicarbazide (2·5 g., recrystallized from methanol) and glycine (0·5 g.) were dissolved in water and made up to 300 ml. after adjusting to pH 9·2 with 2N-NaOH.

Diphosphopyridine nucleotide. This was a 2% (w/v) solution of diphosphopyridine nucleotide-75 (Boehringer und Söhne, Mannheim, Germany).

Alcohol dehydrogenase. Alcohol dehydrogenase suspension (Boehringer und Söhne) was used.

Enzyme preparations

Ram testis. A solution (1%, w/v) of freeze-dried extract of ram testis (obtained from Benger Ltd., Holmes Chapel, Cheshire) was fractionated by addition of saturated ammonium sulphate solution, pH 4·9. The fraction precipitated between 30 and 50% saturation, which contained about 60% of the initial enzyme activity, was taken up in a small volume of water and dialysed for 24 hr. against distilled water at 4°. The activity of this preparation fell by 20% on storage for 4 weeks at 0–4°.

Sweet-almond meal. The meal (obtained through the courtesy of Professor H. N. Rydon) was extracted with 10 ml. of 0·05 M-citrate buffer (pH 4·4)/g. The extract was fractionated as described above except that the fraction precipitated between 30 and 60% saturation with ammonium sulphate was taken. This preparation was extremely stable on storage at 0–4°.

Rat tissues. The freshly dissected tissue was blotted, weighed and then ground with water for 1 min. in a glass homogenizer before diluting to a final concentration of 1% (w/v).

Enzyme estimations

Unit of N-acetyl-β-glucosaminidase. One unit of *N*-acetyl-β-glucosaminidase is defined as the amount of enzyme liberating 1 μmole of phenol from 10 mM-phenyl *N*-acetyl-β-glucosaminide in 0·05 M-citrate buffer, pH 4·4, in 1 hr. at 37°.

Kinetic experiments. Substrate and a suitable dilution of enzyme were incubated at 37° in 0·05 M-citrate buffer. The final volume was 0·4 ml. with ethyl *N*-acetyl-β-glucosaminide as substrate and 1·0 ml. with the aryl glucosaminides. Each estimation was carried out in duplicate. Control tubes, in which enzyme or substrate was omitted, were also incubated. In these experiments substrate concentration, enzyme concentration, pH and duration of incubation were independently varied.

Activity in tissues. The conditions described by Pugh, Leaback & Walker (1957*a*, *b*) were used. The substrate concentrations and periods of incubation were: phenyl *N*-acetyl-β-glucosaminide, 10 mM, 60 min.; *p*-nitrophenyl and *p*-acetylphenyl *N*-acetyl-β-glucosaminide, 3·6 mM, 20 min.

Estimation of liberated aglycone

Phenols. Phenol was estimated as described by Pugh *et al.* (1957*b*); *o*- and *p*-nitrophenol and *p*-acetylphenol were estimated directly in a Unicam SP. 500 spectrophotometer after stopping the enzyme reaction by the addition of 2·0 ml. of 0·25 M-sodium carbonate. The wavelength used was that at which the anion of the phenol showed maximum absorption. The molecular extinction coefficients of the phenols under these conditions are shown in Table 1.

Table 1. *Absorption characteristics of substituted phenols and the corresponding aryl N-acetyl-β-glucosaminides*

Compound	Solvent	$\lambda_{max.}$ (mμ)	$10^{-3} \times \epsilon_{max.}$
p-Nitrophenol	0·1N-HCl	318	9·95
	Na_2CO_3*	400	18·10
p-Nitrophenyl *N*-acetyl-β-glucosaminide	0·1N-NaOH	300	10·80
o-Nitrophenol	0·1N-HCl	280	6·56
	Na_2CO_3*	412	4·60
o-Nitrophenyl *N*-acetyl-β-glucosaminide	0·1N-NaOH	260	3·34
p-Acetylphenol	0·1N-HCl	275	14·10
	Na_2CO_3*	324	22·9
p-Acetylphenyl *N*-acetyl-β-glucosaminide	0·1N-NaOH	265	16·84

* 2·0 ml. of 0·25 M-Na_2CO_3 was added to a solution of the phenol in 1·0 ml. of 0·05 M-citrate buffer, pH 4·4.

The substrates in alkaline solution had absorption bands corresponding to those of the un-ionized phenol, and their extinction coefficients at $\lambda_{max.}$ of the phenol anion were so low that unhydrolysed substrate did not interfere in the spectrophotometric estimation of the phenol liberated in an enzyme assay (cf. Robinson, Smith, Spencer & Williams, 1952). After correcting for enzyme and substrate blanks, the amount of phenol liberated by the enzyme was read from a calibration curve constructed with a standard solution of phenol.

Ethanol. A modification of the method of Bücher & Redetski (1951) was used (cf. Kuhn & Tiedemann, 1954). The enzyme reaction was stopped by the addition of 3·0 ml. of pyrophosphate buffer, pH 9·2. Diphosphopyridine nucleotide (0·1 ml.) and 0·02 ml. of alcohol dehydrogenase were added and, after 1 hr. at room temperature, the tubes were centrifuged at 1500*g* for 15 min. The above-mentioned steps were all carried out in the same stoppered tube. The absorption of the supernatant was measured at 340 mμ in a Unicam SP. 500 spectrophotometer. After correction for the enzyme and substrate blanks, the amount of ethanol liberated by the enzyme was read from a calibration curve constructed from a standard solution of ethanol. Calculation, by using the value $6{\cdot}3 \times 10^3$ for ϵ of reduced diphosphopyridine nucleotide, showed that oxidation of ethanol was 90% complete under these conditions. The calibration curve was reproducible, and ethanol incubated with the enzyme preparations in the absence of substrate was completely recovered.

RESULTS

Effect of pH

The effect of pH on the rate of hydrolysis of each substrate by *N*-acetyl-β-glucosaminidase from testis was determined over the range pH 3·0–5·6. A smooth curve with a single pH optimum was obtained in each case (Table 2). The optimum pH for the hydrolysis of *o*-nitrophenyl *N*-acetyl-β-glucosaminide was higher (4·8) than for the other substrates (4·4–4·5). Similar results were obtained with *o*- and *p*-nitrophenyl *N*-acetyl-β-glucosaminide with the enzyme from sweet-almond meal. The peaks of the curves obtained with this enzyme preparation were broader than those of the corresponding curves for the testis enzyme.

Table 2. *Optimum* pH *for* N-*acetyl-*β*-glucosaminidase*

Activity towards each substrate was determined for seven to ten values within the range pH 3·0–5·5 in 0·05M-citrate buffer. Period of incubation 30 min. For methods of assay see text.

Enzyme preparation	Substrate (*N*-acetyl-β-glucosaminide, mM)	Optimum pH
Testis	Phenyl, 10·0	4·40
	o-Nitrophenyl, 8·0	4·80
	p-Nitrophenyl, 3·6	4·45
	p-Acetylphenyl, 3·6	4·50
	Ethyl, 10·0	4·45
Sweet-almond meal	*o*-Nitrophenyl, 8·0	4·75
	p-Nitrophenyl, 3·6	4·40

Effect of substrate concentration

The effect of various substrate concentrations on the reaction velocity in 0·05M-citrate buffer, pH 4·4, was studied. The results are shown in Table 3. Typical Michaelis–Menten curves for each substrate were obtained by plotting velocity of hydrolysis against substrate concentration. Values for the Michaelis constant (K_m) and theoretical maximum velocity of hydrolysis ($V_{max.}$) were derived graphically by the method of Lineweaver & Burk (1934). Several preparations of each enzyme were used in the course of these experiments. To allow comparison of the results the activity of these preparations was estimated under standard conditions (for definition of units see above) and rates of hydrolysis in Table 3 are expressed as μmoles of substrate hydrolysed/hr./unit of enzyme. At the highest substrate concentrations used the reaction mixtures were approximately half-saturated with respect to glycoside, and the observed rates of hydrolysis were close to the theoretical maximum. There was no indication of inhibition by excess of substrate.

The value for the rate of hydrolysis of each substrate relative to phenyl *N*-acetyl-β-glucosaminide is shown. Ethyl *N*-acetyl-β-glucosaminide was hydrolysed much more slowly than the aryl substrate, whereas the effect of the ring substituent in the phenolic substrates was to increase the rate of hydrolysis.

The sensitivity of a method for the estimation of *N*-acetyl-β-glucosaminidase with one of these substrates depends not only on the rate of hydrolysis of the substrate but also on the extinction obtained per μmole of liberated aglycone in the final spectrophotometric estimation (Table 1) and on the fraction of the incubation mixture available for this estimation (66% for phenyl *N*-acetyl-β-glucosaminide but 100% for the other substrates where no deproteinization is necessary before colour development). The estimation of *N*-acetyl-β-glucosaminidase with *p*-nitrophenyl *N*-acetyl-β-glucosaminide as substrate is some 12 times as sensitive as with the phenyl glycoside.

Competition between substrates

Although Neuberger & Pitt Rivers (1939) showed that *N*-acetyl-β-glucosaminidase hydrolysed both an alkyl and an aryl *N*-acetyl-β-glucosaminide, there is no evidence to show whether the same enzyme is responsible for both these activities. The effect of varied substrate concentrations on the rate of hydrolysis of three aryl *N*-acetyl-β-glucosaminides was studied in the presence and absence

Table 3. *Activity of N-acetyl-β-glucosaminidase towards various substrates*

Activity was measured at five concentrations of each substrate within the range indicated. For methods of assay, evaluation of K_m and $V_{max.}$ and definition of enzyme unit, see text. Relative rates of hydrolysis are referred to the value for phenyl *N*-acetyl-β-glucosaminide.

Enzyme preparation ...	Testis				Emulsin			
N-Acetyl-β-glucosaminide ...	Phenyl	*o*-Nitrophenyl	*p*-Nitrophenyl	*p*-Acetylphenyl	Ethyl	Phenyl	*o*-Nitrophenyl	*p*-Nitrophenyl	*p*-Acetylphenyl
No. of experiments	6	3	4	4	7	4	3	5	3
Range of substrate concentration (mM)	1–10	0·3–10	0·1–4	0·3–7	1–50	1–10	0·2–10	0·1–4	0·1–7
K_m (mM ± S.E.)	2·9 ± 0·1	2·2 ± 0·1	1·5 ± 0·2	1·9 ± 0·1	4·6 ± 0·2	1·1 ± 0·1	0·40 ± 0·02	0·41 ± 0·04	0·51 ± 0·03
$V_{max.}$ (μmoles of substrate hydrolysed/unit of enzyme/hr. ± S.E.)	1·31 ± 0·17	2·60 ± 0·12	3·45 ± 0·10	3·23 ± 0·12	0·13 ± 0·02	1·15 ± 0·11	2·25 ± 0·21	2·55 ± 0·32	2·34 ± 0·10
Relative rate of hydrolysis	1	2·0	2·7	2·5	0·1	1	2·0	2·2	2·1

of a fixed concentration of methyl or ethyl *N*-acetyl-β-glucosaminide. The results, plotted according to Lineweaver & Burk (1934), showed that hydrolysis of the aryl substrates was inhibited competitively by the alkyl compounds. The inhibitor constants (K_i) are shown in Table 4. K_i for ethyl *N*-acetyl-β-glucosaminide agrees well with its K_m as a substrate (Table 3). These results indicate that one enzyme is responsible for the hydrolysis of both aryl and alkyl *N*-acetyl-β-glucosaminides.

N-*Acetyl-β-glucosaminidase in rat tissues*

Dodgson & Spencer (1953) found that several aryl sulphates were unsuitable for the estimation of arylsulphatase in crude tissue preparations, because the phenol liberated by the enzyme underwent further metabolic changes which depended on the nature of the tissue, the substituent group of the phenol and the pH of the reaction mixture. *N*-Acetyl-β-glucosaminidase activities in various rat tissues were compared with phenyl, *p*-nitrophenyl and *p*-acetylphenyl *N*-acetyl-β-glucosaminide as substrates. Recoveries of the phenols added to tissue homogenates under the conditions of assay were 98–102% complete. Table 5 shows that the relative amounts of substrate hydrolysed by each tissue were almost constant. Comparison of the columns in Table 5 shows that the relative activities of tissues measured with any one substrate were closely similar.

Table 4. *Inhibition of hydrolysis of aryl N-acetyl-β-glucosaminides by alkyl N-acetyl-β-glucosaminides*

Various concentrations of each substrate (for ranges see Table 3) were incubated with testis enzyme in the absence and in the presence of the indicated concentration of inhibitor. Each result is the mean of two experiments.

Substrate (*N*-acetyl-β-glucosaminide)	Inhibitor (*N*-acetyl-β-glucosaminide, mM)	K_i (mM)
Phenyl	Ethyl, 6·0	4·8
p-Nitrophenyl	Ethyl, 8·0	5·3
p-Acetylphenyl	Methyl, 14·2	6·7

Table 5. *Activity of N-acetyl-β-glucosaminidase in rat-tissue homogenates estimated with various substrates*

Substrates used were phenyl, *p*-nitrophenyl and *p*-acetylphenyl *N*-acetyl-β-glucosaminide. For conditions of incubation and methods of assay, see text. Results are expressed as μg. of phenol liberated/mg. of tissue (wet wt.)/hr.

	Phenol	*p*-Nitrophenol	*p*-Acetylphenol
Kidney	26·9	108·0	98·5
Liver	6·9	23·8	19·4
Spleen	6·1	22·1	17·2
Testis	3·3	10·8	10·0

DISCUSSION

With a general method for the preparation of *N*-acetyl-β-glucosaminides available, the selection of substrates for examination was based on the work of Williams and co-workers (Spencer & Williams, 1951; Robinson *et al.* 1952) and Dodgson & Spencer (1953) on substrates for β-glucuronidase and arylsulphatase. The advantages in convenience and simplicity of basing the enzyme assay on the direct spectrophotometric estimation of the anionic form of the liberated phenol were demonstrated. The absorption spectra of the substituted phenols and of their glycosides showed that in alkaline solution the absorption of the conjugated phenol was negligible at $\lambda_{max.}$ for the phenol anion. Unhydrolysed substrate did not interfere in the estimation of liberated phenol, and substrate blanks in the assays were low. Dodgson & Spencer (1953) emphasized the additional advantage of using a phenol with anionic $\lambda_{max.} > 310\,m\mu$ so that absorption due to protein is small; a low enzyme blank is achieved without deproteinizing the reaction mixture.

N-Acetyl-β-glucosaminidase acted on alkyl and aryl glycosides, but differences were observed in the Michaelis constants and rates of hydrolysis of the substrates. If K_m is assumed to be an equilibrium constant and its reciprocal a measure of the affinity of enzyme for the substrate, then, in comparison with phenyl *N*-acetyl-β-glucosaminide, the *o*- and *p*-nitro and *p*-acetyl substituents in the aglycone produced similar increases in the rate of hydrolysis of the substrate and in its affinity for the enzyme. Analogous though larger effects on the activity of arylsulphatase towards the corresponding sulphates were observed by Dodgson, Spencer & Williams (1956). Nath & Rydon (1954) obtained similar results in comparing phenyl and *p*-nitrophenyl β-glucosides as substrates for β-glucosidase, but with *o*-nitrophenyl β-glucoside the increase in rate of hydrolysis was much greater. The lower affinity and rate of hydrolysis of ethyl as compared with phenyl *N*-acetyl-β-glucosaminide agrees with the results obtained with other glycosidases (Helferich, Lampert & Sparmberg, 1934). Further information about enzyme–substrate interaction and the nature of the active site is required in order to interpret these results in terms of a reaction mechanism for *N*-acetyl-β-glucosaminidase.

The differences between substrates are, however, relevant to the selection of a method for the estimation of *N*-acetyl-β-glucosaminidase; it was shown that the higher sensitivity and convenience attainable in the spectrophotometric estimation of *p*-nitrophenol or *p*-acetylphenol make their glycosides the substrates of choice. Both phenols were recovered quantitatively after incubation at pH 4·4 with crude tissue preparations (cf. Dodgson & Spencer, 1953).

SUMMARY

1. The hydrolysis of ethyl, phenyl, *o*- and *p*-nitrophenyl and *p*-acetylphenyl *N*-acetyl-β-glucosaminide by preparations of *N*-acetyl-β-glucosaminidase from ram testis and sweet-almond meal has been investigated.

2. The same enzyme is responsible for the hydrolysis of alkyl and aryl *N*-acetyl-β-glucosaminides.

3. The effect of substituents in the benzene ring on K_m and $V_{max.}$ values was studied.

4. *p*-Nitrophenyl *N*-acetyl-β-glucosaminide has been selected as the most convenient and sensitive substrate for the estimation of *N*-acetyl-β-glucosaminidase in crude or purified preparations.

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Studies on Glucosaminidase

3. TESTICULAR *N*-ACETYL-β-GLUCOSAMINIDASE AND *N*-ACETYL-β-GALACTOSAMINIDASE

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After the use of *p*-nitrophenyl *N*-acetyl-β-D-glucosaminide as a substrate for *N*-acetyl-β-glucosaminidase (Borooah, Leaback & Walker, 1961), the substrate *p*-nitrophenyl *N*-acetyl-β-D-galactosaminide has now been used to study the activity of *N*-acetyl-β-galactosaminidase. An extract of ram testis was used in this investigation as a convenient source of *N*-acetyl-β-glucosaminidase. *N*-Acetyl-β-galactosaminidase activity was present in the extract. Comparison of the properties of the two activities in this preparation indicate that both are associated with the same enzyme site.

Preliminary accounts of this work have appeared (Heyworth, Borooah & Leaback, 1957; Woollen, Heyworth & Walker, 1959).

MATERIALS AND METHODS

Substrates and inhibitors. Phenyl and *p*-nitrophenyl *N*-acetyl-β-D-glucosaminide were prepared according to Leaback & Walker (1957) and the corresponding galactosaminides according to Heyworth, Leaback & Walker (1959). The *N*-acetylhexosamines were prepared by the method of Roseman & Ludowieg (1954). *N*-Acetylgalactosamine was chromatographically pure (Heyworth & Walker, 1958). Its aqueous solutions were estimated by the method of Aminoff, Morgan & Watkins (1952) with *N*-acetylglucosamine as the standard. 2-Acetamido-2-deoxygluconolactone and 2-acetamido-2-deoxygalactonolactone were prepared according to Findlay, Levvy & Marsh (1958).

Enzyme preparation. Except where otherwise stated the freeze-dried ram-testis extract (Benger Ltd., Holmes Chapel, Cheshire) was dissolved in aqueous 0·1% crystallized bovine-plasma albumin (Armour Ltd., Hampden Park, Eastbourne, Sussex) (see Results section).

Estimation of enzyme activities. Except where indicated, solutions of enzyme (0·1 ml.) and substrate were mixed in a total volume of 1·0 ml. of 0·05 M-citrate buffer, pH 4·5, and incubated for 30 min. at 37°. Liberated phenol was estimated by the procedure of Pugh, Leaback & Walker (1957). Liberated *p*-nitrophenol was estimated by the method of Borooah *et al.* (1961), modified by the use of 0·2 M-borate buffer, pH 9·8 (Clark & Lubs, 1916), in place of 0·25 M-sodium carbonate. The reasons for this substitution are given below. Phenol did not interfere in the estimation of *p*-nitrophenol under these conditions.

RESULTS

Photolysis of p-*nitrophenyl* N-*acetylhexosaminides in alkaline medium*

In the assay procedure of Borooah *et al.* (1961) there was a slow liberation of *p*-nitrophenol from the substrates after the addition of 0·25 M-sodium carbonate if tubes were exposed to daylight (Fig.1) or u.v. light (365 mμ). No such liberation occurred in the dark or in artificial (tungsten) light. The addition of 0·2 M-borate buffer, pH 9·8, in place of sodium carbonate stopped the enzyme reaction, giving a final pH of 9·2. This pH was sufficiently high for the complete conversion into the anionic form of any *p*-nitrophenol liberated by the enzyme but sufficiently low to cause negligible further breakdown of the substrate even in daylight.

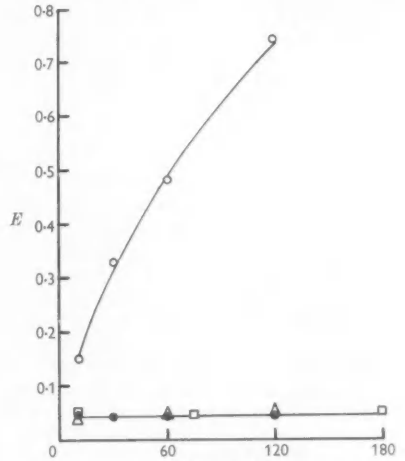

Fig. 1. Photolysis of *p*-nitrophenyl *N*-acetylglucosaminide (2 ml., 3·6 mM in 0·05 M-citrate buffer, pH 4·5) treated with 4 ml. of (1) 0·25 M-sodium carbonate, in daylight (○), in darkness (●), in tungsten light (□), and (2) 0·2 M-borate buffer (pH 9·8) in daylight (△).

Effect of enzyme concentration and time of incubation

The *N*-acetylglucosaminidase activity of a solution of the freeze-dried testis extract (0·1 %, w/v) made in 0·1 % albumin was about 15 % higher than that of a solution made in water (Fig. 2). Activity was not increased further by raising the concentration of albumin to 1 % (w/v). The difference in activity was not due to instability of the enzyme in the absence of added protein under conditions of assay, since both solutions showed constant rates of hydrolysis over periods of incubation up to 1 hr. Fig. 2 shows constant specific activity in the presence of 0·1 % of albumin and a constant absolute loss of activity in the absence of albumin, over a fourfold range of enzyme concentration. Although activity lost on dilution with water was restored by subsequent addition of albumin to the assay mixture, cysteine (4 mM) had no effect and the loss in activity was unaltered with different preparations of the enzyme, substrate, buffer or distilled water; it was therefore not due to contamination of one of the reagents with traces of some toxic impurity (cf. Dixon & Webb, 1958). The loss of activity on dilution could also be prevented by using a solution of the non-ionic detergent Triton X-100 (0·2 %, v/v; Charles Lennig and Co. Ltd., London, W.C. 1) and the effect was therefore probably due to surface inactivation. A similar effect was found with *N*-acetylgalactosaminidase activity.

Effect of pH

Fig. 3 shows the effect of different pH values over the range 3·5–6·0 on the rates of hydrolysis of the two *p*-nitrophenyl substrates relative to the individual maximum activities. *N*-Acetylgalactosaminidase shows a single pH optimum of 4·3, whereas *N*-acetylglucosaminidase shows peaks at pH 4·45 and 4·7. The peak at pH 4·7 was found in two other experiments when albumin was omitted from the assay mixture; it was not found with a partially purified preparation of the testis extract (Borooah *et al.* 1961).

A pH of 4·5 was selected for subsequent experiments, all of which involved comparative measurements of *N*-acetylglucosaminidase and *N*-acetylgalactosaminidase activities.

Effect of substrate concentration

The effect on the rate of hydrolysis by different concentrations of each of the four substrates was measured at pH 4·5 over at least an eightfold concentration range. The K_m and $V_{max.}$ values for the substrates, at pH 4·5, are shown in Table 1. The value of $V_{max.}$ for each *N*-acetylglucosaminide substrate was seven times as great as the value for the corresponding *N*-acetylgalactosaminide. The

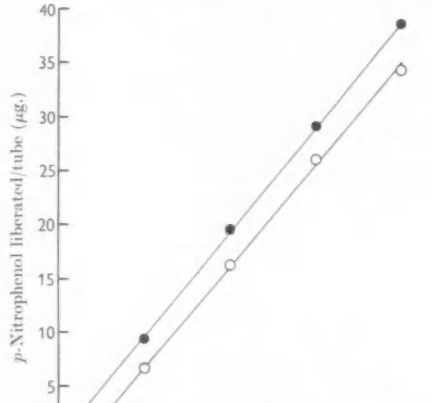

Fig. 2. Effect of enzyme concentration on activity in the absence and presence of albumin. Substrate: 3·6 mM-*p*-nitrophenyl *N*-acetylglucosaminide. The enzyme solutions (0·1 ml./ml. of incubation mixture) were made up in and diluted further with either water (○) or 0·1 % albumin (●). For other conditions see text.

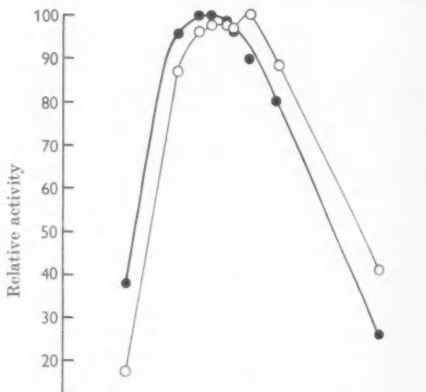

Fig. 3. Effect of pH on *N*-acetylglucosaminidase and *N*-acetylgalactosaminidase activities. Activities are plotted relative to the highest activity (100), with 3·6 mM-*p*-nitrophenyl *N*-acetylglucosaminide and 0·06 % (w/v) of enzyme (○); or 2·2 mM-*p*-nitrophenyl *N*-acetylgalactosaminide and 0·24 % of enzyme (●). For other conditions see text.

Table 1. *K_m and $V_{max.}$ values for substrates at* pH 4·5

Values of K_m and $V_{max.}$ were determined by the method of Lineweaver & Burk (1934). For preparation of enzyme solution and conditions of assay, see text. $V_{max.}$ values are expressed as μmoles of substrate hydrolysed/mg. of freeze-dried testis extract/30 min.

Substrate	Concn. range (mM)	K_m* (mM)	$V_{max.}$* (μmoles/mg. of enzyme)
p-Nitrophenyl *N*-acetylglucosaminide	0·73–5·84	1·18±0·04 (12)	4·07±0·15 (12)
p-Nitrophenyl *N*-acetylgalactosaminide	0·18–1·46	0·28±0·01 (12)	0·59±0·05 (12)
Phenyl *N*-acetylglucosaminide	1·0 –16·0	2·94±0·12 (4)	2·17±0·03 (4)
Phenyl *N*-acetylgalactosaminide	1·0 –16·0	1·22 (2)	0·32 (2)

* Mean ± S.E. (no. of results).

Table 2. *Effect of fractionation of the enzyme on N-acetylglucosaminidase and N-acetylgalactosaminidase activities*

Activities were measured with either 2·2 mM-*p*-nitrophenyl *N*-acetylglucosaminide or *N*-acetylgalactosaminide as substrate. For other conditions see text.

Enzyme preparation	*N*-Acetylglucosaminidase (% of activity of untreated preparation)	Ratio *N*-acetylglucosaminidase/*N*-acetylgalactosaminidase activity
Untreated	100·0	4·6
$(NH_4)_2SO_4$ fractions*		
0–30%	6·1	4·4
30–40%	33·2	4·4
40–50%	15·4	4·4
50–60%	1·5	4·4

* Precipitated between the indicated limits of % saturation with $(NH_4)_2SO_4$.

Table 3. *Adsorption of N-acetylglucosaminidase and N-acetylgalactosaminidase activities by calcium phosphate gel*

Residual activities in the supernatants were measured with either 2·2 mM-*p*-nitrophenyl *N*-acetylglucosaminide or *N*-acetylgalactosaminide as substrate. For other conditions see text.

Total vol. of gel added (ml./10 ml. of enzyme solution)	*N*-Acetylglucosaminidase (% of initial activity)	Ratio *N*-acetylglucosaminidase/*N*-acetylgalactosaminidase activity
0	100	4·9
0·04	89	5·1
0·08	69	5·2
0·12	35	5·0

values of K_m for the *N*-acetylglucosaminide substrates agree with those of Borooah *et al.*(1961) obtained with partially purified testicular extract.

Attempts to separate N-acetylglucosaminidase and N-acetylgalactosaminidase

Fractionation with ammonium sulphate. A solution of the testis extract in water was treated with successive amounts of saturated ammonium sulphate solution at 4°. The precipitated fractions were redissolved in water and dialysed for 3 days at 4° against water. Samples of the original solution and each of the fractions were made 0·1% with albumin and the two activities were measured under standard conditions with 2·2 mM-*p*-nitrophenyl *N*-acetylhexosaminides (Table 2). There was no indication of any separation of the activities.

Fractionation with calcium phosphate gel. An aqueous solution of the enzyme was treated with successive amounts of calcium phosphate gel (Keilin & Hartree, 1951). At each stage a sample of the supernatant was made 0·1% with albumin and the two activities were measured under standard conditions (Table 3). *N*-Acetylglucosaminidase and *N*-acetylgalactosaminidase were adsorbed in stages up to 65% but there was no separation of the two activities.

Partial inactivation. The untreated enzyme and two ammonium sulphate fractions were preincubated at 56° and pH 4·5 for periods up to 6 min. The solutions were then made 0·1% with albumin and the two activities measured (Table 4). *N*-Acetylglucosaminidase and *N*-acetylgalactosaminidase were decreased to about 30% of their initial values without any change in the ratio of the two activities.

Table 4. *Effect of partial inactivation on N-acetylglucosaminidase and N-acetylgalactosaminidase activities*

An untreated enzyme preparation and two $(NH_4)_2SO_4$ fractions (cf. Table 2) derived from it were preincubated in 0·05 M-citrate buffer, pH 4·5, at 56° for the periods indicated. Residual activities were assayed with either 2·2 mM-*p*-nitrophenyl *N*-acetylglucosaminide or 2·2 mM-*p*-nitrophenyl *N*-acetylgalactosaminide as substrate. For other conditions see text.

Enzyme preparation	Time of preincubation (min.)	*N*-Acetylglucosaminidase (% of initial activity)	Ratio *N*-acetylglucosaminidase/*N*-acetylgalactosaminidase activity
Untreated	0	100	4·4
	3	48	4·4
	6	22	4·3
$(NH_4)_2SO_4$ fractions			
30–40%	0	100	4·2
	3	64	4·5
	6	33	4·1
40–50%	0	100	4·4
	3	62	4·4
	6	36	4·2

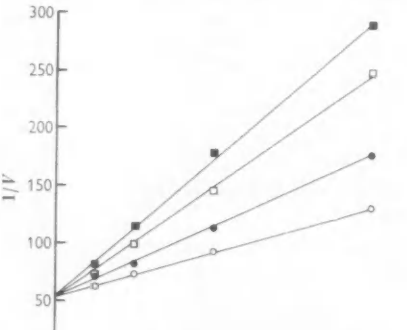

Fig. 4. Effect of different substrate concentrations on the rate of hydrolysis of *p*-nitrophenyl *N*-acetylgalactosaminide. With no addition (○), in the presence of 2 mM-phenyl *N*-acetylglucosaminide (●), 5 μM-2-acetamido-2-deoxygluconolactone (□) and 15 mM-*N*-acetylglucosamine (■). *S*, Substrate concentration (mM); *V*, *p*-nitrophenol liberated/tube (μg.).

Competitive behaviour

N-Acetylglucosamine, 2-acetamido-2-deoxygluconolactone and phenyl *N*-acetylglucosaminide acted competitively with *p*-nitrophenyl *N*-acetylgalactosaminide (Fig. 4). The corresponding galactosamine compounds also competed with this substrate and all six compounds competed with *p*-nitrophenyl *N*-acetylglucosaminide. The similarity of the K_i values, determined by the method of Lineweaver & Burk (1934) and measured against each *p*-nitrophenyl substrate (Table 5), indicates that under these conditions, the compounds com-

Table 5. *K_i values of various competing substances*

Each value is an average of two results, determined by the method of Lineweaver & Burk (1934), from experiments in which competition with (1) *p*-nitrophenyl *N*-acetylglucosaminide (0·73–5·84 mM) or (2) *p*-nitrophenyl *N*-acetylgalactosaminide (0·18–1·46 mM) was studied.

	K_i (mM) (1)	K_i (mM) (2)
Competitive inhibitors		
N-Acetylglucosamine	5·2	6·9
N-Acetylgalactosamine	2·5	2·6
2-Acetamido-2-deoxygluconolactone	0·0037	0·0035
2-Acetamido-2-deoxygalactonolactone	0·0012	0·0017
Competing substrates		
Phenyl *N*-acetylglucosaminide	2·8	3·6
Phenyl *N*-acetylgalactosaminide	0·6	0·8

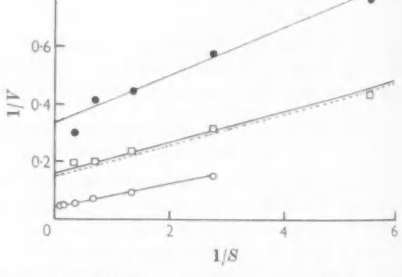

Fig. 5. Effect of different substrate concentrations on the rate of hydrolysis of *p*-nitrophenyl *N*-acetylglucosaminide (○), of *p*-nitrophenyl *N*-acetylgalactosaminide (●) and of equimolar mixtures of the two (□). For conditions see text; *V*, total *p*-nitrophenol liberated (μg.); *S*, total substrate concentration (mM); - - -, theoretical plot for an equimolar mixture of competing substrates derived from the individual substrate results.

pete identically with *p*-nitrophenyl *N*-acetylglucosaminide and *p*-nitrophenyl *N*-acetylgalactosaminide.

Competing substrates

Fig. 5 shows the results obtained in a study of enzymic hydrolysis of the two *p*-nitrophenyl substrates separately and in equimolar mixtures over an eightfold concentration range. The values of K_m and $V_{max.}$ derived from experiments with the individual substrates were used to calculate the theoretical line (dotted) for the total hydrolysis expected from equimolar solutions of two substrates competing for one enzyme site (Thorn, 1949; Foster & Niemann, 1951). The agreement between observed and calculated lines for the mixed substrates was very good.

DISCUSSION

The choice of *p*-nitrophenyl *N*-acetylgalactosaminide as a substrate was based on previous experience with the corresponding *N*-acetylglucosaminide. In spite of its lower rate of hydrolysis, the solubility (0·15 %, w/v) of this substrate and the sensitivity of the estimation procedure of *p*-nitrophenol allowed a sufficiently wide range of substrate concentrations for the accurate determination of $V_{max.}$, K_m and K_i values.

The photolysis of the *p*-nitrophenyl substrates, experienced in daylight when the enzyme reaction was stopped with sodium carbonate, was eliminated by the selection of a buffer of suitable pH and molarity in place of sodium carbonate solution.

The use of 0·1 % albumin solution for diluting the enzyme overcame the loss of specific activity on dilution and the variation in duplicate assays which were experienced in the early stages of this work. Triton X-100 (0·2 %, v/v) had the same protective effect. The behaviour observed was very similar to that shown by highly purified preparations of prostatic acid phosphatase (Tsuboi & Hudson, 1955).

Attempts to separate *N*-acetylglucosaminidase and *N*-acetylgalactosaminidase by fractionation with ammonium sulphate or calcium phosphate gel or by partial heat inactivation were unsuccessful and suggested that the two activities might be associated with the same enzyme protein.

Kinetic experiments were therefore undertaken to test if both activities were associated with one enzyme site. The two *N*-acetylhexosamines, 2-acetamido-2-deoxyhexonolactones and phenyl *N*-acetylhexosaminides behaved competitively with both *p*-nitrophenyl substrates. The similarity of the K_i values for each competitor obtained by measurement against either *p*-nitrophenyl substrate is strong evidence that each substrate (and hence each competitor) is combining with the same point or points on the enzyme surface. This interpretation was confirmed by the good agreement of the experimental plot of the substrate concentration curve for mixed substrates with that calculated on the assumption of competition at a single site.

For two other glycosidases evidence has been obtained that the specificity at C-4 is not absolute. Marsh & Levvy (1958) showed by partial inactivation and by the use of competitive inhibitors that a single enzyme was responsible for the β-glucuronidase and β-galacturonidase activities in preparations from limpet and mammalian tissues. Helferich and his co-workers [for references see Veibel (1950)] failed to separate the β-glucosidase and β-galactosidase activities of almond emulsin either by extensive purification or by various attempts at differential inactivation. Kinetic measurements of the hydrolysis of β-glucosides and β-galactosides seemed to indicate that the two glycosidases were different (Veibel, 1950) but more detailed studies of competition between substrates and of specific inhibition (cf. Conchie & Levvy, 1957) are needed in order to reach a final conclusion.

The lack of specificity of *N*-acetylhexosaminidase for configuration at C-4 is interesting in view of the suggestion that *N*-acetyl-β-glucosaminidase acts on the oligosaccharides formed from hyaluronic acid by the actions of hyaluronidase and β-glucuronidase (Linker, Meyer & Weissman, 1955). Oligosaccharides containing *N*-acetylglucosamine or *N*-acetylgalactosamine are obtained from hyaluronic acid or from chondroitin and chondroitin sulphate by the action of a single enzyme in preparations of testicular hyaluronidase. The corresponding oligosaccharase shows therefore a lack of specificity analogous to that of the polysaccharase.

SUMMARY

1. *p*-Nitrophenyl *N*-acetylgalactosaminide has been used as a substrate to measure the *N*-acetylgalactosaminidase activity of a ram-testis extract.

2. The photolysis of *p*-nitrophenyl *N*-acetylglucosaminide, which occurs in daylight after the enzymic hydrolysis has been stopped by adding sodium carbonate, was overcome by the use of borate buffer, pH 9·8.

3. The presence of 0·01 % of albumin or 0·02 % of Triton X-100 in the enzymic solutions prevented a loss of specific activity on dilution of the enzyme.

4. No separation of the activities of *N*-acetylglucosaminidase and *N*-acetylgalactosaminidase was achieved by fractionation with ammonium sulphate or calcium phosphate gel, or on partial heat inactivation.

5. The K_i values of the competing substances *N*-acetylglucosamine, 2-acetamido-2-deoxyglucono-

lactone and phenyl *N*-acetylglucosaminide and of the corresponding galactosamine derivatives were measured. For each substance the value obtained with *p*-nitrophenyl *N*-acetylglucosaminide as substrate was almost the same as that obtained with *p*-nitrophenyl *N*-acetylgalactosaminide.

6. The competition of *p*-nitrophenyl *N*-acetylglucosaminide and *p*-nitrophenyl *N*-acetylgalactosaminide for one enzyme site was confirmed by an experiment with mixtures of these substrates.

We acknowledge the kind gifts of 2-acetamido-2-deoxygalactonolactone from Dr G. A. Levvy and of freeze-dried ram-testis extract from Benger Ltd. This work was assisted by grants from the Research Fund of the University of London and the Nuffield Foundation.

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Mechanism of Transaminase Action

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It is known that during the course of transaminase action the α-hydrogen atom of an amino acid exchanges with a hydrogen atom of the medium water (Konikova, Dobbert & Braunstein, 1947; Konikova, Kritzmann & Teiss, 1942). We have found that during the reaction an exchange also occurs with the hydrogen atoms at the β position of amino acid. This paper presents the experimental evidence for such an exchange and discusses the significance of the phenomenon for the mechanism of the transaminase reaction. A preliminary report of this work has already appeared (Oshima & Tamiya, 1959).

MATERIALS AND METHODS

Enzyme. An alanine–glutamate transaminase preparation was obtained from pig-heart homogenate as described by Green, Leloir & Nocito (1945). After the third ammonium sulphate precipitation and dialysis, the preparation was freeze-dried and stored at −20°.

Calcium pyridoxal phosphate. Pyridoxal phosphate was prepared from pyridoxamine dihydrochloride according to the method of Wilson & Harris (1951).

α-*Deutero*-DL-*alanine* (DL-[2-^{2}H]*alanine*). α-Deuterium-substituted DL-alanine [$CH_3 \cdot CD(NH_2) \cdot CO_2H$] was prepared by the electrolytic reduction of α-*iso*nitrosopropionic acid [$CH_3 \cdot C(:N \cdot OH) \cdot CO_2H$] in deuterium oxide. The method

of its preparation and the assignment of its infrared-absorption peaks have been described elsewhere (Suzuki *et al.* 1959). Mass-spectrometric analysis showed that the preparation contained 1·0 atom of deuterium per molecule.

Mass-spectrometric analysis. The deuterium content of the compounds was analysed by a modification (Tamiya, 1960) of the method described by Graff & Rittenberg (1952), with a mass spectrometer, model M-60 (Process and Instrument, Brooklyn, New York, U.S.A.).

Infrared-absorption spectra. The infrared-absorption spectra of alanine preparations were recorded by the potassium bromide disk method (thickness, 1 mm.) with a sodium chloride prism in a Koken infrared spectrometer, model DS-301 (Japan Spectroscopic Manufacturing Co. Ltd., Itabashiku, Tokyo, Japan). L-Alanine and α-oxoglutaric acid were obtained from the Nutritional Biochemical Corp., Cleveland, Ohio, U.S.A.

EXPERIMENTAL AND RESULTS

Preparation of αβ-tetradeutero-L-alanine (L-[2-^{2}H:3-^{2}H$_3$]alanine)

L-Alanine (42 mg.), the enzyme preparation (20 mg.), pyridoxal phosphate (0·05 mg.) and α-oxoglutaric acid (0·5 mg.) were dissolved in 99·8% deuterium oxide (5 ml.), containing 0·08 M-phosphate buffer, pH 7·0, made of disodium hydrogen phosphate and potassium dihydrogen phosphate. The final pH of the mixture was adjusted with concentrated sodium hydroxide in 99·8% deuterium oxide to give pH 7·6 (estimated with bromothymol blue). One drop of toluene was added as preservative.

After incubation for 38 hr. at 37° the reaction mixture was boiled and filtered. The amino acid in the filtrate was adsorbed on a column (1·8 cm.^{2} × 7 cm.) of Amberlite IR-112, H type. The column was washed with distilled water and the amino acid eluted with aq. 0·15 N-ammonia solution. The eluate was evaporated and dried under reduced pressure at about 50°; the residue was dissolved again in water (10 ml.) and passed through another column (1·8 cm.^{2} × 3 cm.) of Amberlite IR-45, acetate type, to remove acidic impurities. The solution was then evaporated to dryness under reduced pressure. The alanine thus obtained was dissolved in a minimum amount of water, recrystallized by the addition of ethanol and dried under reduced pressure at 100°. The recovery of alanine was 33 mg., which, as analysed by mass spectrometry, was found to contain deuterium in an amount of 53·7 atoms % of its total hydrogen, or 3·76 atoms of deuterium per molecule. It is evident that these four deuterium atoms were attached to the α- and β-carbon atoms of the alanine, because the other three hydrogen atoms must have readily exchanged with normal hydrogen during the course of isolation.

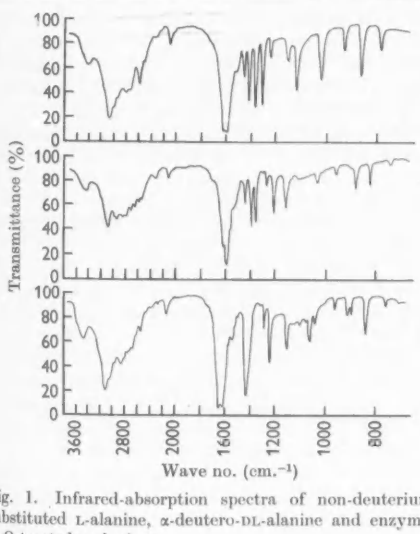


Fig. 1. Infrared-absorption spectra of non-deuterium-substituted L-alanine, α-deutero-DL-alanine and enzyme–D_2O-treated L-alanine.

Fig. 1 shows the infrared-absorption spectra of L-alanine, chemically prepared α-deutero-DL-alanine and enzymically prepared αβ-tetradeutero-L-alanine. It is known that non-deuterium-substituted D-, L- and DL-alanine give the same infrared-absorption spectra. The absorption peak at 1308 cm.$^{-1}$, which is to be assigned to the α-CH deformation (Fukushima, Onishi, Shimanouchi & Mizushima, 1959; Suzuki *et al.* 1959), disappeared in both chemically prepared α-deutero-DL-alanine and the enzyme-treated L-alanine.

A new absorption of α-CD deformation appeared at about 960 cm.$^{-1}$, which corresponded to approx. $1/\sqrt{2}$ of the wave number of the original α-CH absorption. The three absorption peaks at 1451 cm.$^{-1}$, 1356 cm.$^{-1}$ and 1018 cm.$^{-1}$, which were to be assigned to degenerating deformation, symmetric deformation and rocking of the $-CH_3$ group of alanine respectively (Fukushima *et al.* 1959; Suzuki *et al.* 1959), remained unchanged with α-deutero-DL-alanine and they completely disappeared with enzymically prepared αβ-tetradeutero-L-alanine.

The L-alanine, which was recovered from the deuterium oxide medium containing boiled enzyme or no enzyme, was revealed by infrared analysis not to have deuterium incorporated into its molecule.

Recovery of L-alanine from the αβ-tetradeutero-L-alanine

The enzymically prepared αβ-tetradeutero-L-alanine was treated again with transaminase in water to recover L-alanine. αβ-Tetradeutero-L-alanine (10 mg.), the enzyme preparation (5 mg.), α-oxoglutaric acid (0·5 mg.) and pyridoxal phosphate (0·05 mg.) were dissolved in 0·08 M-phosphate

buffer (4 ml.), pH 7·6, made up with water, disodium hydrogen phosphate and potassium dihydrogen phosphate. After addition of one drop of toluene, the mixture was incubated for 10 hr. at 37° and L-alanine (7 mg.) was recovered from the reaction mixture by a method similar to that described above. The recovered alanine showed an infrared-absorption spectrum reproduced in Fig. 2, which indicates the recovery of alanine containing no deuterium.

Comparison of the rates of exchange at α and β positions

L-Alanine (20 mg.) was treated with 99·8% deuterium oxide–phosphate buffer (4 ml.; 0·08 M, pH 7·0) in the presence of transaminase (7 mg.), α-oxoglutaric acid (0·5 mg.) and pyridoxal phosphate (0·05 mg.). The pH of the mixture was adjusted to 7·6 with concentrated sodium hydroxide in 99·8% deuterium oxide. After 10 and 25 min. incubation at 37°, 2 ml. samples of the reaction mixture were withdrawn and the alanine was isolated and purified as described above. The decrease in the absorbance of the infrared peaks at 1308 cm.$^{-1}$ (α-CH deformation) and 1451 cm.$^{-1}$ (–CH_3 degenerating deformation) was compared with that at 1410 cm.$^{-1}$ (–CO_2^- symmetric stretching), which remained unchanged. The absorption at 1451 cm.$^{-1}$ would disappear when only one hydrogen atom in the methyl group was replaced. The results are summarized in Table 1. The values obtained with chemically synthesized α-deutero-DL-alanine and with L-alanine, which had been recovered from the reaction mixture containing boiled enzyme or no enzyme, are also given in the Table. It can be seen that the rate of exchange was about the same at the β position as at the α position. The chemically prepared α-deutero-DL-alanine seemed to contain some of its deuterium at the β position; the amount of β-incorporation increased when the reduction temperature was raised.

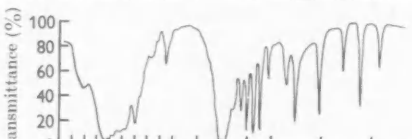


Fig. 2. Infrared-absorption spectrum of recovered non-deuterium-substituted L-alanine.

Table 1. *Incorporation of deuterium into α and β positions of alanine*

L-Alanine (20 mg.) was treated with 99·8% deuterium oxide–phosphate buffer (4 ml.), pH 7·6, in the presence of transaminase (7 mg.), α-oxoglutarate (0·5 mg.) and pyridoxal phosphate (0·05 mg.). After 10 and 25 min. incubation samples (2 ml.) were withdrawn and the alanine was isolated and purified as described in the text. α-Deutero-DL-alanine was prepared by electrolytic reduction of α-*iso*-nitrosopropionic acid in 99·8% deuterium oxide (Suzuki *et al.* 1959).

Alanine preparations	Decrease in absorbance (%) 1308 cm.$^{-1}$ (α – CH)	1451 cm.$^{-1}$ (– CH_3)
10 min. incubation	13	14
25 min. incubation	20	29
16 hr. with boiled enzyme (100°, 3 min.)	2	0
16 hr. without enzyme	0	0
Chemically synthesized α-deutero-DL-alanine	> 90	7

DISCUSSION

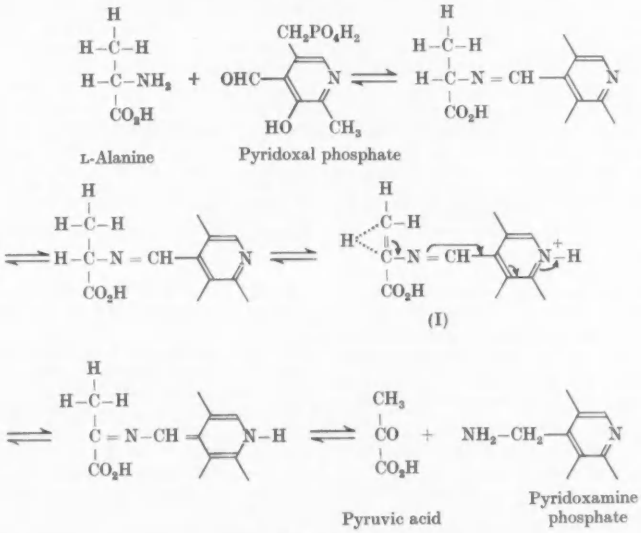
The above-described experiments show that when the transaminase reaction occurred in a medium of heavy water, the hydrogen atoms attached to the β-carbon atom of L-alanine exchanged with the deuterium in the medium. The fact that the rate of exchange at the β position was about the same as that at the α position strongly suggests that the dissociation of a proton from the β-carbon atom is an essential step in the mechanism of transaminase action.

Herbst & Rittenberg (1943) observed an incorporation of deuterium from the heavy water of the medium into the α and β positions of alanine formed in an organic reaction between pyruvic acid and α-aminophenylacetic acid. Taking into consideration the widely accepted idea that the primary step in the transaminase reaction is the formation of a Schiff base from the amino acid and pyridoxal phosphate, the most probable course of events may be pictured as shown in Scheme 1.

According to this Scheme, the Schiff base dissociates a proton from its β-carbon atom, contrary to the prevailing idea that the base dissociates a proton from its α-carbon atom (Schlenk & Fisher, 1947; Braunstein & Shemiakin, 1953; Metzler, Ikawa & Snell, 1954).

The dissociation at the β position is more conceivable than that at the α position also in view of the present knowledge of electronic theory. The dissociation of a proton from the α-carbon atom would be difficult because of the effect of lone-pair electrons on the nitrogen atom, whereas the dissociation from the β-carbon atom may be facilitated by the effect of formation of a hyperconjugated double-bond system in the proposed intermediate.

Almost all the transaminases known to react on amino acids require the presence of at least one hydrogen atom at the β position of their substrate molecules (Meister, 1955). The only exceptions are



Scheme 1

transaminases of glycine and of *tert.*-leucine (Heyns & Koch, 1951). It is also known that the amino acids having electron-withdrawing groups such as carboxyl or phenyl at their β positions are good substrates of transaminases, whereas the amino acids having long side chains at the same position react more slowly than alanine.

The formation of a Schiff base from pyridoxal phosphate and an amino acid is considered to be a common step in various pyridoxal phosphate-dependent enzymic reactions. The β-dissociation concept proposed here may be applicable to most of these reactions, and the compound [designated as (I) in the scheme] may be the key intermediate which undergoes various kinds of reactions.

SUMMARY

1. The four hydrogen atoms attached to the α- and β-carbon atoms of L-alanine exchanged with hydrogen atoms of medium water during the course of transaminase action. The exchange was ascertained by mass spectrometry and infrared spectrometry by following the incorporation and release of deuterium atoms into and from these positions.

2. The rate of exchange at the β position was about the same as that at the α position.

3. Based on the above findings, a new mechanism of transaminase reaction is proposed in which the Schiff base formed from the amino acid and pyridoxal phosphate dissociates a proton primarily from the β-carbon atom rather than from the α-carbon atom. This concept is discussed.

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Condensed Tannins

7. ISOLATION OF (−)-7:3′:4′-TRIHYDROXYFLAVAN-3-OL [(−)-FISETINIDOL], A NATURALLY OCCURRING CATECHIN FROM BLACK-WATTLE HEARTWOOD*

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The interrelated flavonoid compounds (+)-7:3′:4′-trihydroxyflavan-3:4-diol (Keppler, 1957) of 2:3-*trans*:3:4-*cis* configuration (Clark-Lewis & Roux, 1958, 1959), (+)-fustin (2:3-*trans*) and fisetin (Roux & Paulus, 1960) have been isolated from black-wattle-wood tannins. In the present study a new catechin, (−)-fisetinidol, corresponding to these substances has been isolated, and their biogenesis is discussed.

EXPERIMENTAL AND RESULTS

All melting points are uncorrected. Mixed melting points were on equimolecular mixtures of the substances (Roux & Maihs, 1960). Analyses of C, H, methoxyl and acetyl are by Weiler and Strauss, Oxford. Infrared comparisons were by Dr J. R. Nunn, National Chemical Research Laboratory, C.S.I.R., Pretoria.

Isolation of (−)-fisetinidol

Compounds soluble in ethyl acetate from black-wattle heartwood (120 g.) were obtained as described by Roux & Paulus (1960) and redissolved in ethyl acetate (800 ml.). Chloroform (400 ml.) was added slowly with vigorous stirring and the buff-coloured tannin precipitate sucked off and discarded. The filtrate was dried under vacuum in a rotary evaporator. The solid product (41 g.) was extracted with light petroleum to remove residual wax. The wax-free solids (40 g.) were dissolved in 400 ml. of the lower phase of a water–butan-2-ol–petroleum (b.p. 40–150°) (5:3:2) mixture and introduced into the first eight tubes of a 160-tube, 50 ml. underphase, automatic Craig machine (Glasapparatebau Göttingen, Helmut Rettberg). The upper and lower phases of the above mixture were used for countercurrent separation, and after 160 transfers the upper layer of every fifth tube was examined by two-dimensional chromatography on Whatman no. 1 paper with water-saturated butan-2-ol and then aq. 2% acetic acid.

(+)-Fustin (R_F 0·37 in 2% acetic acid on Whatman no. 1 paper) was present in tubes 106–136, and in tubes 106–121 it was accompanied by a second substance of R_F 0·42. The second substance was present also in tubes 96–105 where it was accompanied by what appeared to be a polymeric leuco-fisetinidin of the same R_F as (+)-fustin.

The contents of tubes 96–105 and 106–121 were run off separately, the organic phase was separated, and the aqueous phase extracted with ethyl acetate in each instance. The combined organic and ethyl acetate extracts from each fraction were evaporated to 50 ml. and applied in bands to sheets (5 ml./sheet) of 22½ in. × 18¼ in. Whatman no. 3 chromatographic paper. The chromatograms were developed by upward migration in 2% acetic acid for 12 hr. The fustin (R_F 0·43 on Whatman no. 3 paper), the polymeric leuco-fisetinidin and the (+)-mollisacacidin (traces) (R_F 0·57) bands were located with toluene-*p*-sulphonic acid. Bands in the region R_F 0·50 which appeared yellowish in ordinary light were cut out and eluted with 70% ethanol. Concentrate of the eluents gave 0·113 g. of pale-yellow crystals. The mother liquors were purified by running on five sheets, as above, yielding a further 0·05 g. Pale-yellow crystals of (−)-fisetinidol, m.p. 211–214°, were obtained on recrystallizing from water. They differed in shape from those of (+)-fustin (m.p. 209–211°), of which 1·7 g. was obtained by cutting and eluting the sheets in the region R_F 0·43. The crystals of (−)-fisetinidol did not lose their colour on recrystallizing with charcoal and the m.p. remained unchanged. The substance gave the same colour reactions as (+)-fustin with ammoniacal silver nitrate (grey-black), with bis-diazotized benzidine (yellow) and with ferric alum (green), but treatment with the toluene-*p*-sulphonic acid reagent gave a yellow fluorescence (u.v. light) with (+)-fustin and a yellow colour with the new substance. Microfusion under anhydrous conditions (Roux, 1958*a*) afforded resorcinol and protocatechuic acid only. Infrared-absorption curves over the range 2·5–15 μ showed the absence of carbonyl groups. This suggested that the substance was a catechin (flavan-3-ol) with the same pattern of hydroxylation as (+)-fustin. After drying for 2 hr. at 110° the substance became anhydrous (Found: C, 65·4; H, 5·3. $C_{15}H_{14}O_5$ requires C, 65·7; H, 5·2%) $[\alpha]_D^{25}$ −8·6±0·5° in acetone–water (1:1) (*c*, 0·7).

(−)-7:3′:4′-*Trimethoxyflavan*-3-*ol*. (−)-Fisetinidol (100 mg.) in methanol (20 ml.) was treated with excess of anhydrous diazomethane in ether for 24 hr. at −5°. The solids were recovered by distillation under vacuum in a rotary evaporator, and crystallized from ethanol as fine needles, m.p. 121–123°. $[\alpha]_D^{25}$ −31·9±0·6° in tetrachloroethane (*c*, 0·8) (Found: C, 67·6; H, 6·2; OMe, 30·5. $C_{18}H_{20}O_5$ requires C, 68·3; H, 6·4; OMe, 29·4%).

Synthetic (−)-fisetinidol and derivatives

(−)-*Fisetinidol*. (+)-7:3′:4′-Trihydroxyflavan-3:4-diol [(+)-mollisacacidin] (0·7 g.) was separated from tubes 36–66 of the Craig machine by the same method as above and 550 mg. was hydrogenated in dioxan with palladium catalyst as described by Weinges (1958, 1959). The dioxan

* Part 6: Roux & Paulus (1960).

solution of the flavan-3:4-diol was introduced hot and the apparent absorption, which included contraction on cooling, of 104·2 ml. took place over 11¼ hr. The catalyst was removed by filtration, and the solvent removed under vacuum. The syrup remaining was treated with 15 ml. of water and concentrated to 5 ml., expelling the remaining dioxan. From the solution crystals formed immediately (300 mg.). These were recrystallized from water to give buff transparent crystals, m.p. 211–214°; $[\alpha]_D^{23} - 8{\cdot}8 \pm 0{\cdot}8°$ in acetone–water (1:1) (*c*, 1·1) (Found: C, 65·6; H, 5·3. $C_{15}H_{14}O_5$ requires C, 65·7; H, 5·2%). A mixed m.p. of the natural and the synthetic compounds showed no depression. Their infrared-absorption curves were superimposable over the range 2·5–15 μ.

O-Trimethyl-(−)-fisetinidol. (−)-Fisetinidol (400 mg.) in 80 ml. of methanol was methylated with diazomethane as above. The product crystallized from ethanol (260 mg.) in fine needles, m.p. 121–123°; $[\alpha]_D^{23} - 32{\cdot}1 \pm 0{\cdot}7°$ in tetrachloroethane (*c*, 1·2) (Found: C, 68·3; H, 6·4; OMe, 28·8. $C_{18}H_{20}O_5$ requires C, 68·2; H, 6·4; OMe, 29·4%). A mixed m.p. with the corresponding derivative from black-wattle wood showed no depression. Their infrared curves were identical over the range 2·5–15 μ.

Acetyl-O-trimethyl-(−)-fisetinidol. O-Trimethyl-(−)-fisetinidol (50 mg.) was acetylated with 0·2 ml. of pyridine and 0·2 ml. of acetic anhydride at room temperature for 3 hr. The product was worked up as for tetra-acetyl-(−)-fustin (Roux & Paulus, 1960) and it crystallized as long fine needles from ethanol, m.p. 95–97°; $[\alpha]_D^{23} - 19{\cdot}1 \pm 1{\cdot}5°$ in tetrachloroethane (*c*, 0·6) (Found: C, 67·1; H, 6·1; OMe, 26·5; $CO{\cdot}CH_3$, 10·9. $C_{20}H_{22}O_6$ requires C, 67·0; H, 6·2; OMe, 26·0; $CO{\cdot}CH_3$, 12·0%).

Tetra-acetyl-(−)-fisetinidol. (−)-Fisetinidol (50 mg.) was acetylated with acetic anhydride (0·3 ml.) and pyridine (0·2 ml.) for 3 and for 24 hr. at room temperature. In each instance the product failed to crystallize from methanol, ethanol, ethanol–water and other solvents. The white powder obtained on pouring into water melted over the range 50–60°; $[\alpha]_D^{23} + 0{\cdot}9 \pm 0{\cdot}2°$ in tetrachloroethane (*c*, 1·1) (Found: C, 62·0; H, 4·9; $CO{\cdot}CH_3$, 36·7. $C_{23}H_{22}O_9$ requires C, 62·4; H, 5·0; $CO{\cdot}CH_3$, 38·2%).

Chromatographic comparison of natural and synthetic fisetinidols. (+)-Fisetinidol was prepared, as above, by the hydrogenation of (−)-7:3′:4′-trihydroxyflavan-3:4-diol (Weinges, 1958) from *Schinopsis quebracho-colorado*, and is the enantiomer of (−)-fisetinidol from wattle heartwood and from the hydrogenation of (+)-7:3′:4′-trihydroxyflavan-3:4-diol (cf. Clark-Lewis & Roux, 1959; Roux & Paulus, 1960). These fisetinidols have identical R_F values in partitioning mixtures [0·82 in water-saturated butan-2-ol, and 0·81 in butan-1-ol–acetic acid–water (4:1:5)]. In 2% acetic acid with reference substance (+)-fustin (0·37 on Whatman no. 1 paper) the R_F values were: (+)-fisetinidol synthetic, 0·43; (−)-fisetinidol synthetic, 0·48; (−)-fisetinidol from black wattle, 0·48. This confirms the optical identity of synthetic and natural (−)-fisetinidols. The higher R_F of (−)-fisetinidol in water or 2% acetic acid [compared with (+)-fisetinidol] apparently confirms its stereochemical relationship with (+)-fustin, and (+)-7:3′:4′-trihydroxyflavan-3:4-diol [also (+)-catechin and (+)-gallocatechin], which similarly have higher R_F values in 2% acetic acid than their corresponding enantiomers (Roux & Paulus, 1960).

Table 1. *Radial distribution of (−)-fisetinidol in 50-year-old specimens of* Acacia mollissima

Values are expressed as percentage of dry extract. For methods of extraction see Roux & Paulus (1960).

Location of sample	Tree A	Tree B
Methanol extract		
Heartwood periphery	1·1	0·9
Middle heartwood	0·6	0·9
Central heartwood	0·5	0·7
Combined methanol and acetone–water extracts		
Heartwood periphery	1·1	—
Middle heartwood	0·8	—
Central heartwood	0·7	—
Ethyl acetate extract of whole heartwood	1·1	—

Estimation of (−)-fisetinidol in black-wattle wood

Samples prepared for the estimation of the radial distribution of (+)-mollisacacidin and (+)-fustin (Roux & Paulus, 1960) were also used for the estimation of the radial distribution of (−)-fisetinidol by means of paper chromatography and densitometry (Roux & Maihs, 1960). The (−)-fisetinidol was dissolved in methanol (0·1% soln.) and applied to chromatograms in 10 μg. increments over the range 10–90 μg. to obtain a standard curve for estimation. Ammoniacal silver nitrate spray reagent was used for detection. Results are shown in Table 1.

DISCUSSION

(−)-Fisetinidol from black-wattle heartwood is a new, naturally occurring catechin and the second catechin of the 'resorcinol series' to be isolated. The first catechin of this group, (−)-7:3′:4′:5′-tetrahydroxyflavan-3-ol [(−)-robinetinidol], was isolated by Roux & Maihs (1959, 1960) from the bark of the same tree, *Acacia mollissima*. These substances correspond also in their absolute configuration (see below) to (+)-catechin and (+)-gallocatechin of the 'phloroglucinol series' and may alternatively be designated as 5-deoxycatechin and 5-deoxygallocatechin respectively.

The structure of (−)-7:3′:4′-trihydroxyflavan-3-ol was established as for (−)-robinetinidol by hydrogenation of (+)-7:3′:4′-trihydroxyflavan-3:4-diol in the 4 position under conditions established by Weinges (1958, 1959). The natural and synthetic catechins proved to be identical. The (+)-7:3′:4′-trihydroxyflavan-3:4-diol, occurring naturally in the wood of *A. mollissima* (Keppler, 1957), may be formed from (+)-fustin present in the same wood (Roux & Paulus, 1960) by hydrogenation under conditions established by Freudenberg & Roux (1954) and Roux & Freudenberg (1958). These are therefore stereochemically interrelated.

As the stereochemically interrelated substances (−)-7:3′:4′-trihydroxyflavan-3:4-diol, (−)-fustin

and (+)-fisetinidol have the same absolute configuration as (−)-catechin (Weinges, 1958, 1959; Freudenberg & Weinges, 1959), it follows that their above enantiomers (Clark-Lewis & Roux, 1958, 1959; Roux & Paulus, 1960) have the same absolute configuration as (+)-catechin. The new catechin may therefore tentatively be designated as (2*R*:3*S*)-7:3′:4′-trihydroxyflavan-3-ol (cf. Cahn, Ingold & Prelog, 1956; Freudenberg, 1956; Birch, Clark-Lewis & Robertson, 1957).

The stereochemically interrelated (+)-7:3′:4′-trihydroxyflavan-3:4-diol, (+)-fustin and (−)-fisetinidol (also fisetin) occur in black-wattle wood forming 2·8–13·8 %, 1·3–2·8 % and 0·5–1·1 % respectively of the solids in the acetone–water extract. The (+)-7:3′:4′-trihydroxyflavan-3:4-diol is not only by far the most prominent component, but declines in concentration from the peripheral to the central heartwood. It is difficult to escape the conclusion that the flavan-3:4-diol, (+)-mollisacacidin, is first formed and subsequently undergoes conversion into both (−)-fisetinidol and (+)-fustin, and that the latter is finally also converted into fisetin (Fig. 1). In addition to these interconversions the main transformation in the wood is undoubtedly the conversion of (+)-7:3′:4′-trihydroxyflavan-3:4-diol into a number of leucofisetinidin tannins, as suggested by Roux (1958*b*).

The concept of interconversion and also of condensation into a number of related tannins from a single precursor explains the extreme complexity of condensed tannin mixtures usually found in Nature. The present work also links up with the suggestion by Robinson (1936), supported by Harborne (1960), of a single C_{15} precursor for anthocyanidins based on a flavan-2:3:4-triol structure. By comparison, the present work suggests that catechins (flavan-3-ols), dihydroflavonols (flavan-4-one-3-ols) and flavonols as well as tannins originate from a single flavan-3:4-diol precursor. Anthocyanidins could similarly originate from flavan-3:4-diols by interconversion.

The interconversion apparent in black-wattle wood finds a number of parallels in Nature. For example in *Schinopsis* spp. (quebracho), (−)-7:3′:4′-trihydroxyflavan-3:4-diol the main precursor of quebracho tannins (Roux, 1958*b*, *c*) is apparently accompanied by (−)-fustin (chromatographic evidence) and fisetin. Also in *Acacia melanoxylon*, melacacidin (King & Bottomley, 1954) consisting of two stereoisomeric 7:8:3′:4′-tetrahydroxyflavan-3:4-diols (Roux, 1958*b*), is accompanied by the corresponding flavonol (King & Bottomley, 1954) and 2:3-dihydroflavonol (chromatographic evidence). In *Cotinus coggygria* (syn. *Rhus cotinus*) (−)-7:3′:4′-trihydroxyflavan-3:4-diol is accompanied by (−)-fustin and fisetin (Freudenberg & Weinges, 1959), and in *Robinia pseudacacia* (+)-7:3′:4′:5′-tetrahydroxyflavan-3:4-diol is accompanied by (+)-dihydrorobinetin and

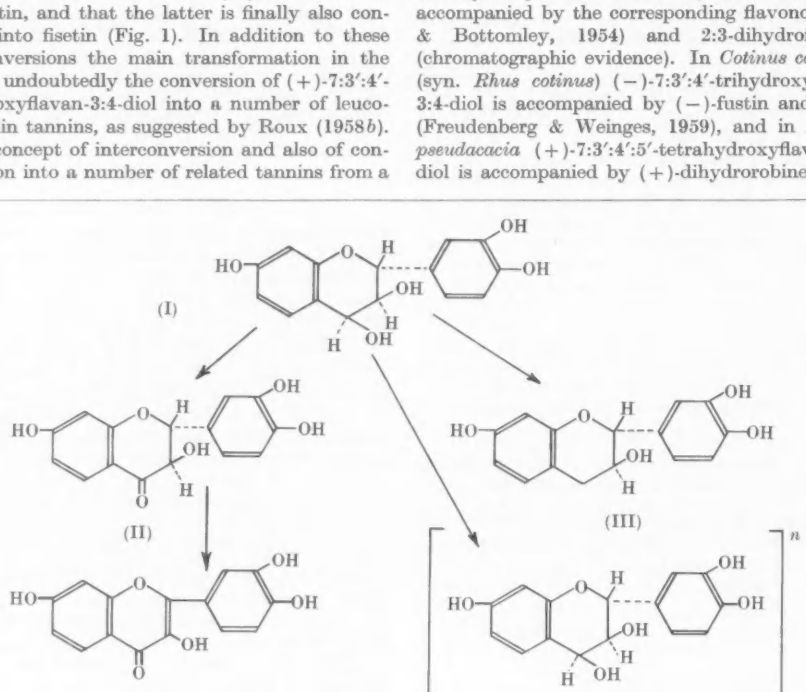

Fig. 1. Suggested formation of tannins, catechins, 2:3-dihydroflavonols and flavonols from the flavan-3:4-diol of wattle wood. (I) (+)-7:3′:4′-Trihydroxyflavan-3:4-diol (2:3-*trans*:3:4-*cis*); (II) (+)-fustin (2:3-*trans*); (III) (−)-7:3′:4′-trihydroxyflavan-3-ol (2:3-*trans*); (IV) fisetin; (V) leuco-fisetinidin tannins.

robinetin (Weinges, 1958). In all these instances the suggested conversion of the flavan-3:4-diol into the corresponding 2:3-dihydroflavonol appears to predominate.

In some woods two flavan-3:4-diol precursors are present, for example 7:4′-dihydroxyflavan-3:4-diol and 7:3′:4′-trihydroxyflavan-3:4-diol in certain *Guibourtia* spp. (Roux, 1959). In black-wattle-bark tannins similarly, two interrelated groups of components are present, namely (*a*) (−)-robinetinidol, robinetin (Kirby & White, 1955), leuco-robinetinidin (traces) and complex leuco-robinetinidin tannins and (*b*) fisetin, leuco-fisetinidin (traces) and complex leuco-fisetinidin tannins. These two groups of substances may arise from the monomeric flavan-3:4-diols by the same paths of interconversion and condensation as is evident for wattle-wood tannins.

SUMMARY

1. (−)-7:3′:4′-Trihydroxyflavan-3-ol [(−)-fisetinidol], a new catechin, has been isolated from the heartwood of *Acacia mollissima*. The radial distribution of the substance in the wood has been estimated.

2. (−)-Fisetinidol has also been prepared synthetically by the hydrogenation of (+)-7:3′:4′-trihydroxyflavan-3:4-diol.

3. The new catechin has the same configuration at C-2 and C-3 as (+)-7:3′:4′-trihydroxyflavan-3:4-diol and as (+)-fustin, all of which occur in the wood.

4. The suggestion is made that the flavan-3:4-diol is the single precursor from which the corresponding catechin, 2:3-dihydroflavonol and flavonol arise by interconversion, and the accompanying leuco-fisetinidin condensed tannins by condensation.

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The Effect of Riboflavin and Mepacrine on the Metabolism of 5-Hydroxytryptamine

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The monoamine-oxidase pathway has been identified as an important route of degradation of 5-hydroxytryptamine (serotonin) in many species. Because of the similarity of its action to that of D-amino acid oxidase, the coenzyme of which is flavinadenine dinucleotide, Richter (1937) suggested that monoamine oxidase may be a flavoprotein. Diamine oxidase has been shown to be a yellow enzyme (Swedin, 1943; Kapeller-Adler, 1949; Goryachenkova, 1956). Hawkins (1952*b*) considered that the decreased activity of monoamine oxidase in the liver of rats, which is a concomitant of riboflavin deficiency, is due to impaired protein utilization and postulated that the vitamin is involved in synthesis of the apoenzyme of monoamine oxidase. Her findings have been

confirmed (Sourkes, 1958). Further evidence of the influence of riboflavin upon monoamine-oxidase activity is the observation that mepacrine, an inhibitor of yellow enzymes, blocks the metabolism of adrenaline *in vivo* and *in vitro* (Allegretti & Vukadinovic, 1950). Mepacrine also inhibits the tetrazolium salt-reducing system of liver suspensions incubated with amine substrates (Lagnado & Sourkes, 1956). These results have led to the tentative classification of monoamine oxidase as a yellow enzyme (Mahler, 1956), but the role of riboflavin in the metabolism of amines requires further clarification. With this aim, the effect of riboflavin deficiency and of mepacrine on the metabolism of 5-hydroxytryptamine has been examined.

MATERIALS AND METHODS

The method of McFarlane *et al.* (1956) was adapted for measurement of 5-hydroxyindolylacetic acid and that of Udenfriend, Weissbach & Clark (1955) for 5-hydroxytryptamine. The experimental animals used were male Sprague–Dawley rats (100–200 g.) and male albino guinea pigs (200 g.). Pooled urine collections were obtained from groups of three animals in plastic metabolism cages (Alpha Plastics Co., Montreal, Canada) for each treatment studied. At the end of the collection period the funnelled base of each cage was washed down with a small volume of water; washings were combined with the urine. Investigations were carried out *in vivo* by administering 2 mg. of 5-hydroxytryptamine/100 g. body wt. to rats by gastric tube; 24 hr.-urine specimens were then collected for determination of 5-hydroxyindolylacetic acid. The urinary output of this acid (above endogenous excretion) was considered a measure of monoamine-oxidase activity. The effect of mepacrine on monoamine-oxidase activity *in vivo* was studied by similarly administering 2 mg. of the drug/100 g. body wt. daily before 5-hydroxytryptamine (California Corporation for Biochemical Research, Los Angeles, Calif., U.S.A.) was given. Riboflavin deficiency was induced by omitting the vitamin from an otherwise fully supplemented artificial diet (Vitamin Test Diet, Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A.). To 1 kg. of the basal diet were added (mg.): thiamine hydrochloride (30); biotin (1·2); calcium pantothenate (80); pyridoxine hydrochloride (30); menadione (4); folic acid (6); 0·1% of vitamin B_{12} triturated in mannitol (10); inositol (2000); *p*-aminobenzoic acid (400); choline chloride (800); powdered sucrose to make 40 g. of mixture. For control animals 30 mg. of riboflavin was included in the vitamin mixture.

Experiments *in vitro* were carried out on livers of riboflavin-deficient rats. These tissues were stored in the frozen state until used, when they were allowed to thaw just before homogenizing in 0·2 M-potassium phosphate buffer, pH 7·4. These homogenates, containing 20% (w/v) of liver, were centrifuged at 20 000 *g* for 30 min. at 2°; the supernatants were removed and respun, and the particulate fractions were washed with buffer, respun and made up to the original volume of the homogenate with buffer. Supernatants were also prepared from fresh guinea-pig livers by the method of Hogeboom (1955) by using 0·25 M-sucrose. Tissue preparations equivalent to 0·5 or 1·0 g. (original wet wt.) of liver were incubated with 2 mg. of 5-hydroxytryptamine in 0·2 M-potassium phosphate buffer, pH 7·4, for 1 hr. at 37° under a flow of $O_2 + CO_2$ (95:5). Final volume was 7·5 ml. After incubation, protein was precipitated with 2·5 ml. of 20% zinc sulphate and the clear supernatant obtained after centrifuging was used for the estimation of 5-hydroxytryptamine and 5-hydroxyindolylacetic acid.

RESULTS

Studies in vivo

The endogenous excretion of 5-hydroxyindolylacetic acid in urine of rats was 0·031 mg./100 g./24 hr. (S.E. 0·007). In many experiments with rats given 5-hydroxytryptamine, amine oxidation, estimated as the increase of 5-hydroxyindolylacetic acid excretion above endogenous, accounted for 3–30% of the administered 5-hydroxytryptamine.

If monoamine oxidase is in some way dependent upon adequate riboflavin nutrition of the animal, it would be expected that riboflavin-deficient rats would metabolize a smaller proportion of exogenous 5-hydroxytryptamine along the monoamine-oxidase pathway than would rats supplemented with the vitamin. The results in Table 1 show that this occurs only occasionally. In fact, the deficient rats often excreted more 5-hydroxyindolylacetic acid than did the control animals. These results cannot be considered definitive

Table 1. *Effect of mepacrine on the excretion of 5-hydroxyindolylacetic acid by riboflavin-deficient rats*

Results are given as mg. of 5-hydroxyindolylacetic acid/100 g. body wt./24 hr., after dosing with 5-hydroxytryptamine (2 mg./100 g.). Male Sprague–Dawley rats on fully supplemented diets were given only enough food to maintain the same body weight as their deficient pairs. Three rats were used for each treatment. Mepacrine, 2 mg./100 g., was administered by gastric tube 24 hr. and 1 hr. before the dose of 5-hydroxytryptamine.

	Set I				Set II			
Days on diet	10	15	20	25	21	25	30	Mean
Treatment with respect to riboflavin:								
Supplemented	0·24	0·20	0·38	0·07	0·25	0·49	0·18	0·26 (100%)
Deficient	0·15	0·23	0·37	0·08	0·40	0·54	0·36	0·30 (115%)
Supplemented + mepacrine	0·33	0·32	0·47	0·44	0·45	0·59	0·30	0·41 (158%)
Deficient + mepacrine	0·34	0·31	0·42	0·19	0·34	0·58	0·38	0·36 (138%)

because of the wide daily variation in output of 5-hydroxyindolylacetic acid by the control animals dosed with 5-hydroxytryptamine, an observation which has been made by others (Erspamer & Ciceri, 1957; Chak & De, 1958). Nevertheless, the results show that riboflavin deficiency does not result in the same change in the oxidation of 5-hydroxytryptamine *in vivo* as observed in extracts of liver, within the same time period, by Hawkins (1952*b*).

It was considered that treatment of the animals with mepacrine, an inhibitor of yellow enzymes (Haas, 1944), would result in a decrease in the amount of 5-hydroxyindolylacetic acid excreted, but it was found that the treatment with mepacrine led to the excretion of a greater proportion of administered 5-hydroxytryptamine as the acidic metabolite (Table 2) than in the controls. This occurred in both riboflavin-supplemented and -deficient animals (Table 1). However, the (weighted) average increase in the former was 60%, compared with only 17% for the deficient rats. This result was checked with the antivitamin, galactoflavin (Emerson, Wurtz & Johnson, 1945) (Merck, Sharpe and Dohme Inc., Rahway, N.J., U.S.A.). In rats receiving a complete diet (Table 3) a single dose of this compound caused an increased output of 5-hydroxyindolylacetic acid (14, 35 and 86% in three experiments). In riboflavin-deficient rats, however, the administration of galactoflavin did not increase the metabolism of exogenous 5-hydroxytryptamine along the monoamine-oxidase pathway but, rather, decreased it.

To assess the value of the 5-hydroxytryptamine-load test for estimating monoamine-oxidase activity in the rat *in vivo* (Tabachnick & Rubin 1959), known inhibitors of monoamine oxidase were studied. Iproniazid (Hoffman–La-Roche Ltd., Montreal, Canada), harmaline and harmine (Dr I. I. A. Tabachnick, Schering Corp., Bloomfield, N.J., U.S.A.) and tranylcypromine (SKF *trans* 385) (Smith, Kline and French Labs., Phil., Pa., U.S.A.) all decreased the excretion of 5-hydroxyindolylacetic acid (Table 4).

Table 2. *Effect of mepacrine on excretion of 5-hydroxyindolylacetic acid by rats dosed with 5-hydroxytryptamine*

All animals (three for each treatment) received a dose of 5-hydroxytryptamine (2 mg./100 g. body wt.) before collection of urine for 24 hr. Mepacrine, 2 mg./100 g. body wt., was administered daily by gastric tube.

Treatment	Days on treatment	5-Hydroxyindolylacetic acid in urine (mg./100 g. body wt./24 hr.)	5-Hydroxyindolylacetic acid in urine (% of control)
None	2	0·13	100
Mepacrine		0·18	138
None	4	0·17	100
Mepacrine		0·23	135
None	7	0·17	100
Mepacrine		0·22	129
None	11	0·11	100
Mepacrine		0·13	118

Table 3. *Effect of galactoflavin on excretion of 5-hydroxyindolylacetic acid by rats dosed with 5-hydroxytryptamine*

Galactoflavin, 2 mg./100 g. body wt., was administered intragastrically 1 hr. before the dose (2 mg./100 g. body wt.) of 5-hydroxytryptamine. Riboflavin-supplemented animals were pair-weighted to the deficient animals, as in Table 1.

Treatment	5-Hydroxyindolylacetic acid in urine (mg./100 g. body wt./24 hr.)	5-Hydroxyindolylacetic acid in urine (% of control)
None	0·43	100
Galactoflavin	0·49	114
None	0·26	100
Galactoflavin	0·35	135
Riboflavin-supplemented	0·07	100
Riboflavin-supplemented + galactoflavin	0·13	186
Riboflavin-deficient	0·08	114
Riboflavin-deficient + galactoflavin	0·05	71

Studies in vitro

Monoamine oxidase is mainly associated with the particulate fraction of rat livers (Hawkins, 1952*a*). Both particulate and supernatant fractions were allowed to act upon 5-hydroxytryptamine at pH 7·4 for 1 hr. at 37°, when the 5-hydroxytryptamine remaining was measured. Much pigment was formed in both fractions. From Table 5 it is seen that the riboflavin deficiency did not appreciably affect the rate of disappearance of the amine in the particulate fractions. Incubation of the amine with the supernatant fraction, however, led to a 25% decrease in the loss of amine under the conditions used. This suggests that the enzyme systems of the supernatant fraction differ from those associated with the cellular residue and, by extension, from monoamine oxidase.

According to Weissbach, Redfield & Udenfriend (1957) the liver of guinea pig contains a soluble monoamine oxidase located in the supernatant fraction. This preparation has the advantage that it contains an excess of aldehyde dehydrogenase capable of increasing formation of 5-hydroxyindolylacetic acid and inhibiting pigment production. This tissue was used to determine whether the two systems acting in rat liver could be distinguished

Table 4. *Effect of monoamine oxidase on excretion of 5-hydroxyindolylacetic acid by rats dosed with 5-hydroxytryptamine*

Dose of 5-hydroxytryptamine (2 mg./100 g. body wt.) was administered by gastric tube. Other compounds were given by gastric tube (p.g.) or intraperitoneally (i.p.).

Treatment	Dose (mg./100 g. body wt.)	Route of administration	5-Hydroxyindolylacetic acid in urine (mg./100 g. body wt./24 hr.)	5-Hydroxyindolylacetic acid in urine (% of control)
Control			0·18	100
Iproniazid	15	p.g.	0·02	11
Control			0·17	100
Iproniazid	15	p.g.	0·05	34
Control			0·41	100
Iproniazid	7·5	p.g.	0·22	54
Control			0·32	100
Iproniazid	7·5	p.g.	0·06	19
Control			0·22	100
Harmaline	1	i.p.	0·02	9
Tranylcypromine	1	p.g.	0·01	4
Control			0·32	100
Harmine	0·5	i.p.	0·21	66

Table 5. *Effect of riboflavin deficiency on disappearance of 5-hydroxytryptamine* in vitro

Rat-liver preparations were used. The conditions are described under Methods.

Days on diet	5-Hydroxytryptamine disappearing (mg./g. wet wt. of liver): Particulate, Riboflavin-supplemented	Particulate, Riboflavin-deficient	Supernatant, Riboflavin-supplemented	Supernatant, Riboflavin-deficient
14	1·89	1·72	1·42	1·26
20	1·44	1·22	1·23	0·77
21	1·64	1·73	1·38	1·00
Mean	1·66	1·56	1·34	1·01

here. For this purpose, the supernatant fraction of fresh guinea-pig liver was incubated with 5-hydroxytryptamine and both disappearance of the amine and formation of 5-hydroxyindolylacetic acid were estimated in each sample. The difference (calculated as 5-hydroxytryptamine) served to estimate the extent of metabolism of the amine along pathways not involving monoamine oxidase. By varying the substrate concentration it was found that the enzymes of the monoamine-oxidase pathway (monoamine oxidase itself and an aldehyde dehydrogenase) were saturated by about 575 μM-5-hydroxytryptamine and that metabolism of the amine along other pathways continued at a linear rate beyond this concentration. Mepacrine, which had been shown in rats to increase the proportion of administered amine appearing in the urine as 5-hydroxyindolylacetic acid, was now tested on this system. Guinea pigs were treated with mepacrine, 20 mg./kg., for 4 days. A final dose was given 1 hr. before the animals were killed and their livers excised. The results are shown in Fig. 1. It can be seen that the treatment of the animals with mepacrine led to a decrease in the rate at which 5-hydroxytryptamine was metabolized along the other pathways, but had no effect upon the formation of 5-hydroxyindolylacetic acid *in vitro*. In experiments with the supernatant fraction of guinea-pig liver pigment formation was not observed.

DISCUSSION

The action of monoamine oxidase converts 5-hydroxytryptamine into 5-hydroxyindolylacetaldehyde, a compound which has not been isolated. Its semicarbazone (prepared from the aldehyde, as it is formed enzymically) turns yellow with hydrochloric acid, and measurement of the specific absorption of this derivative has been used in the assay of monoamine oxidase (Weissbach *et al.* 1957). In the present work the test of monoamine-oxidase activity *in vivo* has depended upon the further oxidation of this aldehyde to 5-hydroxyindolylacetic acid in the tissue. On this basis the findings demonstrate that in the rat the monoamine-oxidase pathway is not the major metabolic

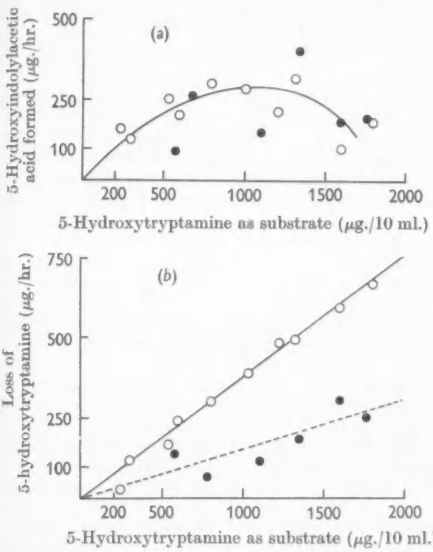

Fig. 1. Effect of mepacrine *in vivo* on metabolism of 5-hydroxytryptamine by guinea-pig-liver supernatants. (*a*) Formation of 5-hydroxyindolylacetic acid; (*b*) loss of amine by pathways not involving monoamine oxidase. ○, Control; ●, with mepacrine. For conditions, see Methods section.

route of 5-hydroxytryptamine when given intragastrically, for less than one-third of the amine appeared in the urine as 5-hydroxyindolylacetic acid. It is possible that the action of monoamine oxidase is actually greater than revealed by these measurements. For example, a portion of the aldehyde formed may be converted, not into the acid, but into other products not determined by the method used. Furthermore, it is possible that reduction of the aldehydic product of oxidation to the corresponding alcohol occurs, as has been observed with *iso*amylamine, phenylethylamine, tyramine and, possibly, tryptamine (Guggenheim & Löffler, 1916) and catecholamines (Kopin & Axelrod, 1960). However, this reaction has not been demonstrated with 5-hydroxytryptamine. Diversion of aldehyde into pigment formation through condensation reactions (Blaschko & Hellman, 1953) also occurs; as yet there is no accurate method for estimating the immediate product of the action of monoamine oxidase upon the amine. The aldehyde dehydrogenase responsible for the conversion of 5-hydroxyindolylacetaldehyde into 5-hydroxyindolylacetic acid is a flavoenzyme and would presumably undergo some diminution in activity in the course of riboflavin deficiency. However, this result would be measured in the present studies only as a net effect on the formation of the acid and would not be reckoned among the other enzyme systems.

The present experiments do not provide evidence that riboflavin is involved in the action of monoamine oxidase in the same sense as it is in that of D-amino acid oxidase. Riboflavin-deficient rats did not show a decreased excretion of 5-hydroxyindolylacetic acid after administration of 5-hydroxytryptamine as might have been expected from the decreased oxidation of this amine by the livers of such animals (Hawkins, 1952*b*; Sourkes, 1958). The fact that excretion of the acid could be increased by two riboflavin antagonists, mepacrine and galactoflavin, suggests that a more complex mechanism than monoamine oxidase alone is operative and that these drugs bring about the diversion of a greater proportion of the exogenous 5-hydroxytryptamine into the monoamine-oxidase pathway than normally, through blocking some other route of degradation of the amine. This other pathway can be assumed to be a 'normal' one, the activity of which is diminished by flavoenzyme inhibitors, as well as by riboflavin depletion (seen in the supernatant fraction of deficient rat livers).

This hypothesis of a second riboflavin-dependent enzyme involved in the metabolism of 5-hydroxytryptamine explains the results of Tables 2 and 3. Thus when the metabolism of the amine has already been diverted from this enzyme into the monoamine-oxidase route, because of riboflavin deficiency, then the effect of mepacrine or of galactoflavin is less than that seen in the riboflavin-fed controls. The disappearance of 5-hydroxytryptamine from supernatant fractions of rat liver was decreased by 25% as a result of riboflavin deficiency, whereas the decrease in particulate fractions containing the major portion of monoamine oxidase (Hawkins, 1952*a*) amounted to but 6%. Because clear supernatant fractions are not obtained with stored tissues later homogenized in phosphate buffer, some activity measured in this fraction was undoubtedly due to monoamine oxidase, but hardly enough to account for the observed change in activity. The evidence adduced from studies of liver supernatant obtained from guinea pigs treated with mepacrine indicates that mepacrine is acting under these conditions as an inhibitor of an enzyme or of a system of enzymes apart from monoamine oxidase.

SUMMARY

1. The effect of mepacrine and of riboflavin deficiency on 5-hydroxytryptamine metabolism has been studied *in vivo* and *in vitro*.

2. In rats dosed with the amine, prior treatment with mepacrine increased excretion of 5-hydroxy-

indolylacetic acid. Riboflavin deficiency frequently had the same effect.

3. Mepacrine did not affect formation of the acid in the supernatant fraction of homogenized guinea-pig liver but it decreased the disappearance of the amine along other pathways.

4. Riboflavin deficiency resulted in decreased disappearance of the amine in supernatants of rat liver but not in the particulate fractions.

5. A hypothesis has been proposed that in mammalian tissue there is a pathway of metabolism of 5-hydroxytryptamine, other than the one catalysed by monoamine oxidase, which is sensitive to flavoenzyme inhibition and to riboflavin deficiency.

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Evidence for the Occurrence of Uridine Diphosphate *N*-Acetylglucosamine in Crustacean Tissues

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The polysaccharide chitin, which, from available evidence, is believed to consist of unbranched chains of *N*-acetylglucosamine residues, is a prominent constituent of the arthropod exoskeleton. Although information about chitin biosynthesis in arthropods is very sparse, its formation in *Neurospora crassa* involves uridine diphosphate *N*-acetylglucosamine as a glycosyl donor (Glaser & Brown, 1957). Although this and other uridine coenzymes are utilized extensively in the tissues of higher animals, relatively little is known of their biochemistry in invertebrates. This paper describes an investigation of the nucleotide content of the hypodermis and hepatopancreas of three species of Crustacea, with particular reference to the possible function of uridine diphosphate derivatives in polysaccharide biosynthesis.

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MATERIALS AND METHODS

Animals. Live specimens of the shore crab *Carcinus maenas* and of the spider crab *Maia squinado* were obtained from the Marine Biological Laboratory, Plymouth. Live lobsters (*Homarus vulgaris*) were obtained from local dealers.

Extraction of nucleotides from hepatopancreas and hypodermal tissue. (i) Perchloric acid method. The freshly excised tissue was weighed, frozen in liquid oxygen, powdered in a cold mortar and then dispersed in 2 vol. of cold 0·6N-perchloric acid. The insoluble residue was collected by filtration and re-extracted with cold 0·6N-perchloric acid. The combined filtrates were neutralized with 5N-KOH and the precipitated $KClO_4$ was removed by centrifuging, the neutralized extract being diluted to 5 ml. final volume for every gram of tissue used.

(ii) Aqueous extraction. The tissue was cooled on ice, weighed and minced in a chilled blender (a MSE Nelco 10 homogenizer or a Waring Blendor was used). The suspen-

sion was boiled for 2 min. and, after filtration, the residue was re-extracted with boiling water. The combined filtrates were centrifuged and had a final volume of 10–20 ml./g. of tissue.

Phenol treatment of extracts. Coloured matter in *Carcinus* extracts was removed by phenol extraction by the procedure of Storey & Dutton (1955).

Precipitation of barium salts of nucleotides. An extract was freed of glycogen by addition of an equal volume of ethanol and centrifuging. Nucleotides were precipitated from the supernatant by addition of a 20% (w/v) solution of $BaCl_2,2H_2O$ (0·5 ml./2·5 ml. of extract) followed by 4 vol. of ethanol. The solution was adjusted to pH 9 and cooled to −14°; the barium salts were collected by centrifuging, dissolved in cold 0·1 N-HCl and re-precipitated from cold, alkaline, 80% ethanol as before. Barium was removed by dissolving the final precipitate in the minimum quantity of cold 0·1 N-HCl and treating the solution either with Amberlite IR-100 (K^+), or with saturated K_2SO_4 solution and centrifuging; if necessary, the final solution of nucleotides was neutralized with 5 N-KOH.

Ion-exchange chromatography on Dowex 1. Dowex 1 X2, 200–400 mesh, was used for all experiments. Nucleotide mixtures were fractionated with the acid and neutral formate gradient systems of Hurlbert, Schmitz, Brum & van Potter (1954), and by stepwise elution from chloride columns (Cabib, Leloir & Cardini, 1953). Mixtures containing 5–10 μmoles of total nucleotides were resolved on columns 0·5 cm. diam. × 15 cm. high; for gradient elution with the formate systems, the formate solution passed from a reservoir to the column through an airtight system of two flasks in series, each containing 100 ml. of water initially, and magnetically stirred; 5 ml. fractions were collected. The gradient produced by systems of this type is described by Drake (1955). Mixtures containing larger amounts of total nucleotides were fractionated on columns 0·9 cm. diam. × 10–30 cm. high. Formate columns were run in the cold room (4°) and chloride columns at room temperature.

Nucleotides in formate-column effluents were rendered salt-free by concentration on a rotary evaporator, followed by freeze-drying (0·1–0·5 mm. Hg) at room temperature to remove formic acid and ammonium formate. Solutions of nucleotides containing chloride were concentrated and made salt-free by adsorbing the nucleotides on to unground Nuchar C charcoal followed by elution with 50% ethanol containing 1–2% (v/v) of NH_3 soln. (sp.gr. 0·880) (Cabib *et al.* 1953). Ammonia and ethanol were removed on a rotary evaporator. In some cases this charcoal treatment was applied to original tissue extracts to remove pigments.

For identification of nucleotides in column effluents, the pooled fractions corresponding to a given peak were desalted, concentrated and, in some cases, further purified by paper chromatography, with the neutral ammonium acetate–ethanol solvent of Paladini & Leloir (1952). Portions of the concentrated fractions were hydrolysed with 2 N-HCl in sealed tubes at 100° for 6 hr. and the free bases liberated were identified by paper chromatography in propan-2-ol–HCl–water (Wyatt, 1951), with authentic purines and pyrimidines as markers. The ratio total phosphate/base was also measured.

Compounds separated on Dowex-1 formate columns were identified by measurement of the ratio E_{275}/E_{260} and by reference to data for calibrated columns (Hurlbert *et al.* 1954). In preliminary formate separations, and with chloride-column fractionations, further confirmation was obtained by identification of the free bases and measurement of the total phosphate/base ratio.

Phosphate estimations. The methods of Chen, Toribara & Warner (1956) and of Berenblum & Chain (1938), with the modification of Bartley (1953), were employed. For estimation of total phosphate, samples were concentrated and ashed by the method of Burton & Petersen (1960). 'Labile phosphate' corresponded to that released by N-HCl at 100° in 20 min.

N-Acetylamino sugar. This was estimated by the borate method of Reissig, Strominger & Leloir (1955), and by the carbonate method of Aminoff, Morgan & Watkins (1952).

Hydroxamate tests. The following modification of the method of Peel (1951) was used. Immediately before use, an alkaline solution of hydroxylamine was prepared by mixing equal volumes of 2·5 N-NaOH and 2 M-hydroxylamine–HCl. To the sample contained in 0·5 ml. of water was added 1 ml. of alkaline hydroxylamine solution; after 15 min. at room temperature or at 37°, 0·25 ml. of 5·6 N-HCl, 0·25 ml. of $FeCl_3$ solution (15%, w/v, of $FeCl_3,6H_2O$ in 0·2 N-HCl) and 1·0 ml. of water were added. The solution was mixed thoroughly and the extinction at 530 mμ was measured against a 'blank' containing no ester. With ethyl acetate, a linear increase in extinction was obtained in the range 0·1–1·0 μmole.

Prostatic phosphomonoesterase and Russel viper-venom diesterase. These were purified preparations kindly given by Dr G. B. Petersen. For incubations of nucleotides with these enzymes the procedures of Burton & Petersen (1960) were followed.

Spectrophotometric measurements. For the range 350–850 mμ a Unicam SP. 600 spectrophotometer was generally used. For some measurements in this range, and all measurements in the ultraviolet range, a Unicam SP. 500 or a Beckman model DU spectrophotometer was used. The extinctions of nucleotide solutions were measured against water in quartz cells of 1 cm. light path.

In calculating the quantities of nucleotides present in solutions, use was made of the molar-extinction coefficients quoted by Beaven, Holiday & Johnson (1955). To estimate the approximate amounts of nucleotides in crude extracts, the extinction at 260 mμ was measured and a value of 10 used for the millimolar extinction coefficient.

Paper chromatography. This was carried out in all-glass tanks with Whatman no. 1 paper. When quantitative estimations of nucleotides were required the paper was washed in 2 N-acetic acid, followed by six changes of water.

Nucleotides were chromatographed in the following solvent systems: neutral or acidic (pH 3·8) ammonium acetate–ethanol (Paladini & Leloir, 1952); *iso*-butyric acid–NH_3 (Krebs & Hems, 1953); propan-1-ol–NH_3 soln. (sp.gr. 0·880)–water (6:3:1, by vol.; Hanes & Isherwood, 1949).

Sugars were chromatographed in butanol–ethanol–water (4:1:5, by vol.), phenol saturated with water or ethyl acetate–pyridine–water (2:1:2, by vol.).

Detection. Ultraviolet-absorbing compounds were located either by visual inspection under ultraviolet light or by making contact prints with unfiltered light from a mercury lamp (Markham & Smith, 1949). Reducing sugars were detected with the aniline hydrogen phthalate spray of Partridge (1949). *N*-Acetylamino sugars were located with

Table 1. *Nucleotide content of tissue extracts*

Abbreviations used: CMP, cytosine monophosphate; AMP, adenosine monophosphate; GMP, guanosine monophosphate; UMP, uridine monophosphate; ADP, adenosine diphosphate; UDP, uridine diphosphate; ATP, adenosine triphosphate. The exact identities of the compounds designated 'AMP' were not determined, but they were presumed to be isomers of AMP with possibly some adenine diphosphopyridine nucleotide.

Species	Tissue	Extraction method	Preliminary purification procedure*	Total nucleotides eluted (μmoles/g.)	Nucleotides eluted (μmoles/g.)								
					CMP	'AMP'	GMP	UMP	ADP	UDP	UDPX	ATP	Unidentified
H. vulgaris	Hepatopancreas	Perchloric acid	Ba pptn. IR-100	0·74	0·203†		0·011	0·059†		0·25	0·22	0	0
	Hepatopancreas	Perchloric acid	Charcoal	1·19	0·37†		0·04	0·07†		0	0·32	0·39	0
	Hepatopancreas	Perchloric acid	Charcoal Ba pptn. IR-100	0·4	0·19†		0·04	0·02†		0	0·15	0	0
	Hepatopancreas	Perchloric acid	Ba pptn. IR-100	0·93	0·17†		0·18	0·08†		0	0·50	0	0
	Hepatopancreas	Water then perchloric acid	Ba pptn. IR-100	6·98	1·04	1·0	2·38	1·31	0·15	0	0·75	0·22	0·13
	Hepatopancreas	Water then perchloric acid	Ba pptn. IR-100	5·5	0·96	0·99	1·74	0·77	0·10	0	0·65	0·16	0·13
	Hypodermis	Perchloric acid	Ba pptn. IR-100	0·2	0·08	0·03	0	0	0·01	0	0·08	0	0
	Hypodermis	Perchloric acid	Ba pptn. K_2SO_4	0·14	0·06	0·01	0	0	0	0	0·07	0	0
	Hypodermis	Water	Ba pptn. IR-100	1·13	0·39†		0·09	0	0·48	0	0·15	0·022	0
C. maenas	Hypodermis	Perchloric acid	Ba pptn. IR-100	0·11	0·08†		0	0	0	0	0·03	0	0
	Hypodermis	Perchloric acid	Ba pptn. IR-100	0·02	0·01†		0	0	0	0	0·01	0	0
	Hypodermis	Water	Charcoal	1·75	0		0·19	0·18	0·58	0	0·27	0·08	0·45
	Hypodermis	Water	Phenol extraction Ba pptn. IR-100	2·22	0·71†		0·13	0·28	0·26	0·20	0·23	0·08	0·33

* For details see Methods.

† Complete resolution not attained.

the modified Elson and Morgan spray reagent (Partridge, 1948), and free (non-*N*-acetylated) amino sugars and amino acids with ninhydrin (0·4% in butan-1-ol saturated with water).

Paper electrophoresis. This was carried out at room temperature in a Perspex apparatus made by Shandon, London. Whatman no. 1 paper (8 cm. × 32 cm.) held between two glass plates was used.

Reference nucleotides. Uridine diphosphate *N*-acetylglucosamine, uridine 3′-monophosphate and uridine 5′-monophosphate were obtained from the Sigma Chemical Co., St Louis, Mo., U.S.A.

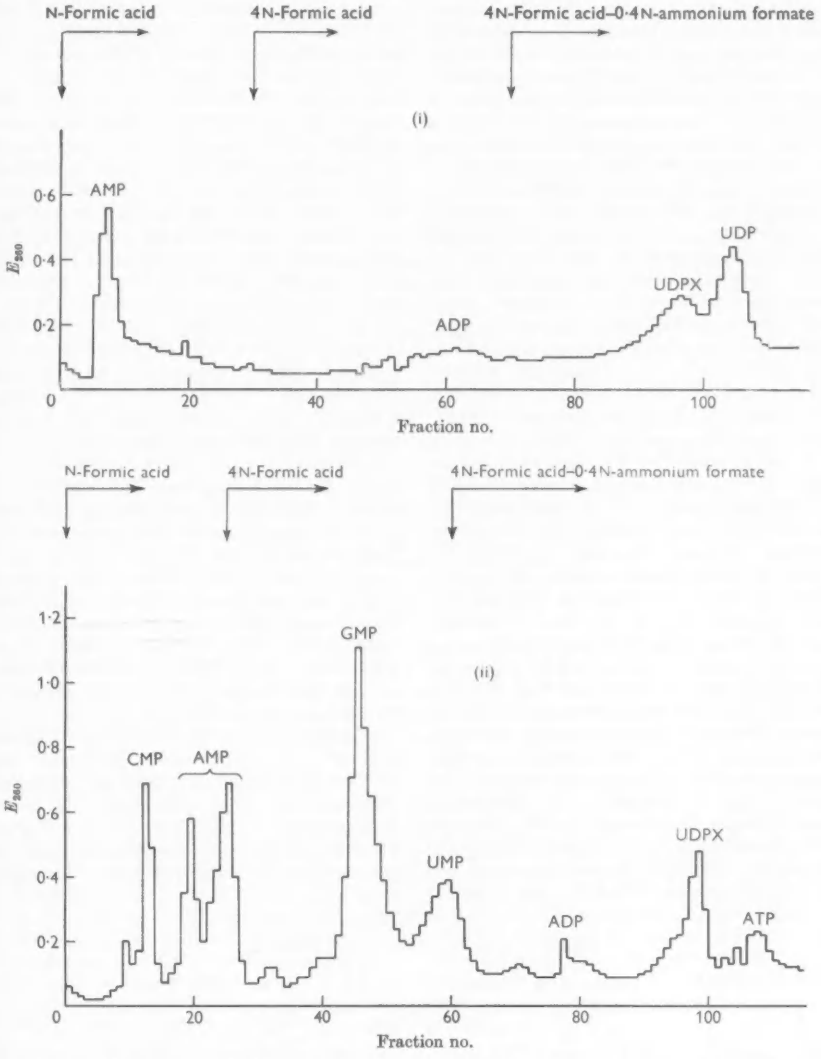

Fig. 1. Dowex-1 fractionation of lobster-hepatopancreas extracts. Resin columns 15 cm. high × 0·5 cm. diam. with the acid formate-eluting system of Hurlbert *et al.* (1954) were used. (i) Total barium salts from 3·6 g. wet wt. of tissue extracted with cold perchloric acid. (ii) Total barium salts from 1·3 g. wet wt. of tissue extracted with boiling water.

RESULTS

Nucleotide content of tissue extracts

Considerable difficulty was experienced in obtaining reproducible column chromatograms of tissue extracts, especially those of *Carcinus* hypodermis. A comparison of various extraction and purification procedures was therefore carried out in order to determine the most favourable conditions for obtaining high yields of nucleotides.

Fractionation by column and paper chromatography of the hypodermis and hepatopancreas extracts revealed the presence of mixtures of nucleotides, including *N*-acetylhexosamine derivatives of uridine diphosphate. The results of a number of experiments are summarized in Table 1, and a comparison between two experiments is given in Fig. 1. In all cases where the methods were compared, extracts made with hot water contained a wider range and larger quantities of nucleotides per g. wet weight of tissue than those prepared by extraction with perchloric acid. (Expression of the nucleotide contents on a wet-weight basis was only satisfactory for hepatopancreas tissue, which can be easily weighed before extraction; lobster hypodermis always retained some fragments of muscle after the necessarily rapid dissection, and *Carcinus* hypodermis was difficult to remove without a quantity of fluid simultaneously draining out of the carapace. The recorded wet weights of hypodermal tissues are therefore high, but serve as a rough guide within the series of experiments with a particular species.) Purification of the nucleotides via their barium salts and removal of the barium either with Amberlite IR-100 or as $BaSO_4$ made little difference to the overall yields. With the heavily pigmented aqueous extracts of *Carcinus* hypodermis, purification of the nucleotides as their barium salts was inadequate for obtaining satisfactory fractionations on Dowex-1; in these cases it was found that preliminary treatment with phenol or charcoal was essential, although experiments with other tissue extracts indicated that appreciable losses of nucleotides may occur in such cases. The qualitative differences in nucleotide contents for the two extraction methods were reasonably consistent for all the tissues. Perchloric acid extracts usually contained a high proportion of the amino sugar-containing nucleotides designated UDPX, with cytidine monophosphate and adenosine monophosphate as the other main constituents; only traces of uridine monophosphate and guanosine monophosphate, with some adenosine diphosphate, uridine diphosphate and adenosine triphosphate, were usually detected. By contrast, in aqueous extracts the proportion of UDPX was of the order 10–12 %, with corresponding increases in the other constituents. Nucleoside triphosphates, with the exception of adenosine triphosphate, were not detected in significant quantity in any of the extracts examined. It was concluded that with these tissues extraction with hot water provides a very much more efficient means of obtaining nucleotides than does treatment with cold perchloric acid. The reasons for this difference were not determined.

Structural investigations on N-acetylamino sugar nucleotide fractions

Homogeneity of compounds. After isolation by ion-exchange chromatography, the nucleotides were rechromatographed on Dowex-1 with eluting systems different from those used in the original separations; the homogeneity with respect to ultraviolet-absorbing material was then further checked by paper chromatography. The compounds corresponded to those designated UDPX in Table 1, and also included similar substances isolated from the hypodermis of *M. squinado* and the hepatopancreas of *C. maenas*. Since the same results were obtained with each of the nucleotides isolated from the five different tissues, it seemed likely that these compounds were identical; the structural investigations are therefore described in terms of a single substance.

In each experiment the compound possessed a typical uridine ultraviolet-absorption spectrum which was abolished by bromine in acid solution (Caputto, Leloir, Cardini & Paladini, 1950); hydrolysis with 2N-HCl at 100° for 6 hr. yielded uracil. Two molecules of phosphate were present per molecule of uridine (Table 2). Hydrolysis with 0·1N-HCl at 100° for 30 min. released uridine

Table 2. *Properties of UDPX nucleotides*

Source of nucleotide ...	*Carcinus maenas*		*Homarus vulgaris*		*Maia squinado*
	Hypodermis	Hepatopancreas	Hypodermis	Hepatopancreas	Hypodermis
Uridine	1·0	1·0	1·0	1·0	1·0
Total phosphate	1·98	2·08	1·96	1·89	1·74
'Labile phosphate'	0·99	—	—	1·19	—
N-Acetylamino sugar					
Borate method	1·0	1·16	1·0	0·91	0·91
Carbonate method	0·84	1·23	0·98	0·62	—

5′-phosphate, which was identified by paper electrophoresis in 0·1 M-borate buffer at 7 v/cm. for 6–7 hr. (Crestfield & Allen, 1955), and by paper chromatography in neutral ammonium acetate–ethanol, with authentic uridine 5′-phosphate and uridine 3′-phosphate as markers. Hydrolysis with 0·01 N-HCl at 100° for 5 min. released uridine diphosphate, which was identified chromatographically by comparison with the product obtained from uridine diphosphate *N*-acetylglucosamine under the same conditions of hydrolysis (Cabib *et al.* 1953).

A positive Morgan and Elson reaction was obtained only after hydrolysis with 0·01 N- or 0·1 N-HCl at 100°, the substance giving the positive test coinciding with the ultraviolet-absorbing areas on paper chromatograms. Calibrated for *N*-acetylglucosamine, the borate method indicated a 1:1 ratio for *N*-acetylamino sugar/uridine, the spectrum of the chromogen closely resembling that given by *N*-acetylglucosamine (Table 2). There was no increase in this apparent ratio when the carbonate method of Aminoff *et al.* (1952) was used, and the chromogen's spectrum again resembled that given by *N*-acetylglucosamine. It was therefore concluded that stable 3-*O*-substituted derivatives of *N*-acetylglucosamine were absent (Cifonelli & Dorfman, 1957). Hydrolysis with 2 N-HCl at 100° for 2 hr. produced a substance which moved with glucosamine and gave positive tests for amino sugars on paper chromatograms. Ninhydrin treatment of this amino sugar produced arabinose (Stoffyn & Jeanloz, 1954), indicating that the parent sugar was either glucosamine or mannosamine. To distinguish between these two possibilities, the products of hydrolysis of the nucleotides with 0·1 N-HCl were chromatographed on borate-treated paper with butan-1-ol–pyridine–water (6:4:3, by vol.), which is reported to separate *N*-acetylglucosamine and *N*-acetylmannosamine (Comb & Roseman, 1958); the modified Elson and Morgan spray reagent revealed only a compound moving with *N*-acetylglucosamine. With a number of other solvent systems, only glucosamine or *N*-acetylglucosamine was detected on paper chromatograms of acidic hydrolysates of the nucleotide.

To test for amino acids, the compound was treated with 6 N-HCl at 100° for 15–18 hr. and the hydrolysates were examined by paper chromatography (phenol saturated with water). Preparations of the nucleotide from lobster and *M. squinado* hypodermis were never obtained completely free from traces of amino acids, although none was detected in the purified substance isolated from the other three sources.

Hydroxamate tests for carboxylic esters in the intact nucleotide gave negative results.

Treatment of the compound with snake-venom diesterase followed by phosphomonoesterase released a substance which gave a positive Morgan and Elson test and which moved with *N*-acetylglucosamine on paper chromatograms.

Chromatographic behaviour of the nucleotide. In early experiments, certain preparations of the crustacean amino sugar-containing nucleotide, although closely resembling uridine diphosphate *N*-acetylglucosamine in its reactions, appeared to differ from it chromatographically (Kent & Lunt, 1958). However, it has subsequently been found that the nucleotide did not separate from uridine diphosphate *N*-acetylglucosamine when co-chromatographed with the latter on paper, or on chloride columns, although high salt concentrations (0·06 N-NaCl in 0·01 N-HCl) were required for elution. By contrast, during the preparation of a sample of uridine diphosphate *N*-acetylglucosamine from pig liver, it had been found, in agreement with Cabib *et al.* (1953), that uridine diphosphate *N*-acetylglucosamine required only 0·03 N-NaCl in 0·01 N-HCl for displacement from Dowex-1 chloride columns.

It was concluded that the actual chloride concentration required for elution did not necessarily provide a reliable means of identification of nucleotides on ion-exchange chromatography, although the order of appearance of compounds in column eluates proved to be reproducible under the conditions described above.

From these results it was concluded that each crustacean amino sugar-containing nucleotide was probably uridine diphosphate *N*-acetylglucosamine.

DISCUSSION

The most frequently investigated form of chitin is that of crab and lobster, where with proteins it forms the basis of the organic matrix of the exoskeleton. Biosynthesis of this tissue is believed to occur in the hypodermis, a thin cellular layer underlying the cuticle itself. However, comparatively little information is available about the biochemical activities of the hypodermis, the study of which is complicated by its high content of phenolic substances. There are indications in this tissue of *N*-acetyl-β-glucosaminidase and chitinase (Kuhn & Tiedman, 1954; Lunt & Kent, 1960), but although these enzymes may be significant in the reabsorption of chitin, it appears unlikely that they possess any significant biosynthetic function (Strominger, 1960).

The present studies show that the hypodermis contains a number of ribose nucleotides, including the monophosphates of cytidine, uridine, guanosine and adenine, and the diphosphates of adenine and uridine, as well as adenosine triphosphate. In

the hypodermis of each of the species investigated there is, however, only one amino sugar-containing nucleotide, as judged by ion-exchange and paper-chromatographic procedures. Degradation of this product yielded only uridine monophosphate, phosphoric acid and *N*-acetylglucosamine and it is considered to be uridine diphosphate *N*-acetylglucosamine or a closely allied substance. It therefore seems probable that the formation of the chitin of the exoskeleton occurs through the intermediary of this nucleotide, as with *Neurospora crassa* (Glaser & Brown, 1957). This nucleotide is present in appreciable quantity in the hepatopancreas of the lobster (0·15–0·75 μmole/g. wet wt. of tissue) and of *C. maenas* (0·22 μmole/g. wet wt. of tissue) and has been isolated from the hypodermis of the crab *M. squinado*.

The quantitative and qualitative yields of nucleotides varied according to the extraction procedures used, hot water being superior to cold perchloric acid in these experiments. With heavily pigmented tissues, substances which interfere with chromatographic procedures could be removed by phenolic extraction or by adsorption of the nucleotides on to charcoal, followed by elution with ethanolic ammonia solutions.

SUMMARY

1. Methods for the extraction and chromatographic fractionation of ribonucleoside phosphates from crustacean tissues are described.

2. Evidence is presented for the occurrence in these tissues of a uridine diphosphate amino sugar nucleotide, which is probably identical with uridine diphosphate *N*-acetylglucosamine.

3. The nucleotide is present in appreciable quantities in the hypodermis and hepatopancreas of *Carcinus maenas* and *Homarus vulgaris*, and in the hypodermis of *Maia squinado*.

We are grateful to Professor Sir Hans Krebs, F.R.S., for his interest and to Dr Margery G. Ord and Dr L. A. Stocken for their helpful advice. We also thank the Trustees of the Christopher Welch Scholarships and the Medical Research Council for their awards to one of us (M. R. L.) during the early part of this work. The work was aided by a grant from the Rockefeller Foundation.

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Studies in Immunochemistry

18. THE ISOLATION AND PROPERTIES OF A SIALOMUCOPOLYSACCHARIDE POSSESSING BLOOD-GROUP Le^a SPECIFICITY AND VIRUS-RECEPTOR ACTIVITY*

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Werner (1953) and Odin (1959) examined in considerable detail the different types of carbohydrate–protein complexes occurring in human epithelial mucus, in secretions of the digestive and respiratory tracts, in cervix uteri and especially in ovarian-cyst fluids, and distinguished two main forms of carbohydrate bound to protein, glycoprotein and mucoprotein or sialomucin.

The glycoproteins isolated from these sources were found to be closely similar in chemical and physical properties to the blood-group-specific substances, materials called neutral mucopolysaccharides, which had previously been isolated from human secretions and tissue digests and thoroughly examined (cf. Kabat, 1956; Morgan, 1947, 1959). The blood-group A, B, H and Le^a mucopolysaccharides earlier described each contained four sugars, fucose, galactose, glucosamine and galactosamine, which made up about 80% of each substance, and not less than 11 or 12 firmly bound amino acids, which together comprised the remaining 20% or so of the mucopolysaccharide. The substances sometimes contained a relatively small amount of sialic acid.

The second type of carbohydrate–protein combination called sialomucin, mucoprotein or, more simply, muco-substance, contained much less carbohydrate and a larger amino acid-containing moiety than did the neutral mucopolysaccharide. The sugar components, however, were different, and in place of fucose, which was decreased in amount to about 1% and was possibly there as mucopolysaccharide impurity, sialic acid was present as a major component. These muco-substances were found to be widespread in the body tissues and fluids, more especially in urine (Tamm & Horsfall, 1952; Gottschalk, 1952; Odin, 1952; Tamm, Bugher & Horsfall, 1955), in the pseudomyxomatous water-insoluble gelatinous mucus of ovarian, peritoneal and appendiceal origin (Odin, 1959), and in serum (Weimer, Mehl & Winzler, 1950; Winzler, 1955). The present views on mucopolysaccharides and on sialo- or muco-proteins are reviewed by Bettelheim-Jevons (1959).

* Part 17: Buchanan, Crumpton & Morgan (1957).

Muco-substances which contain fucose and sialic acid as major components were believed to be present in cervical mucus (Shettles, Dische & Osnos, 1951; Bergman & Werner, 1951; Werner, 1953), and in the mucus of certain intermediate-type cystadenoma (Odin, 1959), but, until recently, substantially homogeneous, water-soluble materials containing considerable amounts of both these sugars had not been described. Kawasaki (1959), however, isolated from cancerous and normal gastric mucosae, mucopolysaccharides which were closely similar in composition and blood-group activity to the neutral mucopolysaccharides described above but contained, in addition, up to 10% or so of sialic acid. Gibbons (1959) has also obtained from mid-cycle and from pregnancy cervical mucus of cattle, mucoid materials which contain considerable amounts of sialic acid, but which are otherwise similar in type to the human-blood-group substances.

A mucopolysaccharide which contains fucose and sialic acid as major constituents as well as galactose, both amino sugars and at least 11 or 12 amino acids, has now been isolated from a single human ovarian-cyst fluid, and is described in the present paper. In the absence of any agreed terminology, the substance is described as a sialomucopolysaccharide. A preliminary communication on this material has already appeared (Pusztai & Morgan, 1960).

MATERIALS AND METHODS

Cyst fluid. Obtained from a pseudomucinous ovarian cystadenoma, volume 4 l. The patient's blood phenotype was $A_1Le(a+)$.

Nitrogen. Kjeldahl nitrogen was determined in the apparatus of Markham (1942) and with bromocresol green–methyl red indicator (Ma & Zuazaga, 1942).

Methylpentose. The method of Dische & Shettles (1948), as modified by Gibbons (1955), was employed. Fucose standards (10–50 μg.) were included in each determination.

Amino sugars. A modification (Rondle & Morgan, 1955) of the method of Elson & Morgan (1933) was used. Glucosamine hydrochloride standards in the range 10–50 μg. were included in each determination. Extinctions were read at 535 mμ and the results expressed as glucosamine base.

Galactosamine gives at this wavelength about 92% of the absorption given by an equal weight of glucosamine.

Individual sugars. The quantitative determination of each sugar was also made by paper chromatography with a procedure closely similar to that described by Wilson (1959). The mixtures of sugars obtained after hydrolysis of the mucopolysaccharide, in sealed ampoules with 0·5 N-HCl at 100° for 18 hr., were freed from acid by shaking with Amberlite IR-4 B resin in the carbonate form and concentrated *in vacuo*. The individual sugars were separated on paper with, as solvent mixture, ethyl acetate–pyridine–acetic acid–water (7·5:5:1:3) and developed with aniline hydrogen phthalate reagent (Partridge, 1949). Each coloured area was cut out and cut into small strips, and the strips were placed in a test tube and extracted with 8 ml. of 0·7 N-HCl in 80% (w/v) ethanol, as described by Leloir (1951). Extinctions were determined in 2 cm. cells in a Unicam spectrophotometer. Fucose and galactose (60–70 μg.) were measured at 390 mμ and the two amino sugars (120–150 μg.) at 420 mμ. These analyses were kindly carried out for us by our colleague, Dr D. Doyle.

Reducing sugars. Determined by the colorimetric technique of Nelson (1944). The reducing values are expressed as glucose.

Sialic acid. Determined by the resorcinol method (Svennerholm, 1957), a specimen of *N*-acetylneuraminic acid, kindly supplied by Professor Blix, being used as standard. Absorption was measured at 450 and 580 mμ to eliminate the interference caused by other sugars in the mucopolysaccharide. A preparation of the mucopolysaccharide which had been derived from the sialomucopolysaccharide by removal of the sialic acid by mild acid treatment was included in the assay, and served as a control for the influence of the sugars present on the sialic acid determination. Extinction values obtained were subtracted from those obtained for the sialomucopolysaccharide. *N*-Acetylneuraminic acid standards from 20 to 100 μg. were included in each determination.

The total sialic acid was also determined by the thiobarbituric acid method (Warren, 1959) after the sialomucopolysaccharide had been heated at 80° in 0·1 N-H_2SO_4 for 1 hr. to liberate the sialic acid.

Physical examination. Ultracentrifugal and electrophoretic examinations were carried out at pH 8·0, as described by Kekwick (1950).

Preparative ultracentrifugal experiments were made in a Spinco ultracentrifuge (model L) at 1–2°.

Ultraviolet-light absorption was determined on a Hilger quartz spectrophotometer, with an optical path of 1 cm.

Relative viscosity was measured at 20° at a concentration of 1% (w/v) in water in an Ostwald-type viscosimeter.

The intrinsic viscosities were determined from measurements made at 20° in phosphate buffer (I 0·2) containing 0·15 M-NaCl, pH 8.

Determination of serological activity. The specific Le^a activity was measured as described by Grubb & Morgan (1949) and Annison & Morgan (1952), with a human anti-Le^a serum Aalerud, kindly supplied by Dr J. Lundevall, and group O Le^a+ cells. A standard Le^a substance was included in all tests. The substance had no A, B or H activity (Race & Sanger, 1958).

Inhibition of viral haemagglutination. Serial dilutions of 0·1% solution of the substance in 0·9% sodium chloride solution were mixed with an equal volume of unheated or heated (56° for 30 min.) virus equivalent to about four haemagglutinating doses. The mixtures were allowed to stand at room temperature for 15 min., a 1% (v/v) suspension of fowl cells (0·1 ml.) was added to each tube, and after standing for 75 min. the degree of inhibition was read as described by Belyavin, Westwood, Please & Smith (1951).

EXPERIMENTAL AND RESULTS

Isolation of a sialomucopolysaccharide

A part of the cyst fluid was dried from the frozen state and extracted with 90% (w/w) phenol, as described by Morgan & King (1943) and by Gibbons, Morgan & Gibbons (1955). The phenol-insoluble residue was freed from phenol by prolonged dialysis and a small amount of water-insoluble material which separated during dialysis was removed by centrifuging. The clear viscous solution of the phenol-insoluble, water-soluble material was adjusted to about 1% (w/v) concentration and saturated with $(NH_4)_2SO_4$. The solution, which remained clear, was warmed with constant stirring in the presence of a slight excess of solid $(NH_4)_2SO_4$. A sticky precipitate began to form between 45° and 50°, and separated completely after heating at about 60° for 5 min. As the solution cooled, the particles of the precipitate coalesced and changed to a visco-elastic mass which attached itself firmly to the bottom of the vessel and to the stirrer, and could be easily collected. The material that was insoluble in cold saturated $(NH_4)_2SO_4$ was washed by trituration with saturated $(NH_4)_2SO_4$ solution and was dissolved in water, and the process of saturating with $(NH_4)_2SO_4$, heating to 60° and collecting the precipitate formed, was repeated. About 5% only of the original phenol-insoluble water-soluble material was soluble in saturated $(NH_4)_2SO_4$ at 60°. The material insoluble in saturated $(NH_4)_2SO_4$ was dissolved in water (2%, w/v), potassium acetate (2%, w/v) was added and the substance was fractionated by the addition of ethanol. Fractions separated between 48 and 55% (v/v) of ethanol (24·6 g.), 55 and 57% (v/v) of ethanol (2·4 g.) and 57 and 65% (v/v) of ethanol (2·8 g.), and accounted for almost the whole of the substance insoluble in saturated $(NH_4)_2SO_4$. The fractionation process was repeated on each of the materials and the substances finally obtained were thoroughly dialysed against distilled water at 1–3° and dried from the frozen state. Except where otherwise stated, the observations which are described below were made on the material isolated and purified as described, and finally again precipitated from 1% (w/v) solution in water by ethanol between 48 and 55% concentration.

Properties of the sialomucopolysaccharide

General. The material dissolved readily to give 2% (w/v) solutions in water or in 0·9% sodium chloride solution which were viscous, clear and colourless. Qualitative chromatographic examination of the products of hydrolysis with 0·5N-HCl at 100° for 18 hr. showed the preparation to contain fucose, galactose, glucosamine, galactosamine and, in a 6N-HCl hydrolysate, at least 11 or 12 amino acids. The material gave a strong colour reaction (Svennerholm, 1956) for sialic acid; P was absent.

Physical. The mucopolysaccharide that was insoluble in cold saturated $(NH_4)_2SO_4$ immediately after it had been precipitated at 60° was, after it had been recovered by dialysis and drying from the frozen state, readily soluble in this solvent. The material (0·1% in water) showed no specific absorption bands between the wavelengths 220 and 320 mμ. The optical rotation was *laevo*, $[\alpha]_D - 24°$ for the main fraction (Table 1).

The material examined electrophoretically migrated anodically as a single substance (Fig. 1*a*), substantially free from materials with a different

Table 1. *Analytical values for mucopolysaccharide obtained from ovarian-cyst fluid (no. 350)*

The dried cyst fluid was extracted with (*a*) 90% phenol, (*b*) further fractionated with saturated $(NH_4)_2SO_4$ at 60° and finally precipitated from aqueous solution by ethanol.

	(*a*) Material insoluble in phenol, soluble in water				
		(*b*) Insoluble in saturated $(NH_4)_2SO_4$ at 60°			Soluble in saturated $(NH_4)_2SO_4$ at 60°
Ethanol fraction (%) ...		48–55	55–57	57–66	50–55
Nitrogen (%)	4·9	4·8	4·8	4·8	4·5
Fucose (%)	8·3	8·4	7·6	7·2	10·5
Hexosamine (%)*	27·0	26·6	25·7	25·1	28·4
Reducing sugar (%)*	51·0	51·3	50·2	48·4	54·0
Sialic acid (%)†	14·6	19·0	—	—	10·6
Sialic acid (%)‡	14·0	17·9	18·1	19·4	10·1
Relative viscosity (η)	2·02	2·88	2·32	1·64	1·26
$[\alpha]_D$ in water (*c*, 1%)	−24°	−24°	−17°	−17°	−31°

* After hydrolysis with 0·5N-HCl for 16 hr. at 100°.
† As *N*-acetylneuraminic acid (resorcinol method).
‡ As *N*-acetylneuraminic acid [Warren's (1959) method].

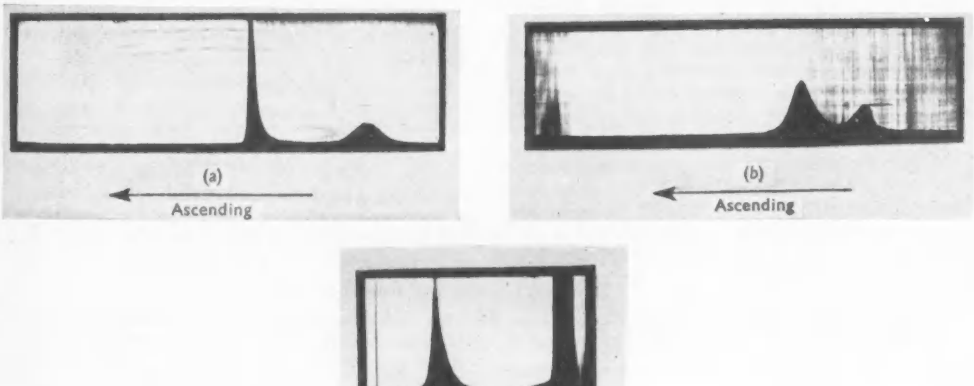

Fig. 1. (*a*) Electrophoretic pattern of sialomucopolysaccharide (ascending boundary) after 120 min. at 5 v/cm. of 2% (w/v) solution in phosphate, pH 8, *I* 0·2. (*b*) Mucopolysaccharide derived from the sialomucopolysaccharide after hydrolysis with acid at pH 2 and 37° for 24 hr.; conditions of electrophoresis were as in (*a*). Arrows show direction of migration in (*a*) and (*b*). (*c*) Sedimentation diagram of sialomucopolysaccharide after 90 min. at 270 000 g; 1·1% (w/v) in phosphate–NaCl (phosphate, pH 8, *I* 0·2; NaCl, 0·15M).

electrophoretic mobility. The rate of migration, determined by Dr R. A. Kekwick, from the position of the descending boundary after 4·5 hr., was 7·5 cm.^{2} sec.$^{-1}$ v^{-1} × 10^{-5} at pH 8.

The relative viscosity of a 1 % (w/v) solution in water of the phenol-insoluble water-soluble material, and those of the materials insoluble and soluble in saturated $(NH_4)_2SO_4$ obtained from it, are given in Table 1. The highest viscosity is shown by the material insoluble in saturated $(NH_4)_2SO_4$ at 60°.

The intrinsic viscosity of the substance insoluble in saturated $(NH_4)_2SO_4$ was determined by a linear extrapolation to zero concentration of η spec./c values plotted against the concentration (Table 2).

The rate of sedimentation of the material observed in the ultracentrifuge showed (Fig. 1*c*) the preparation to contain only a single main component, which appeared to be somewhat polydisperse. The dependence of the sedimentation constant (S) on concentration is evident from the values given in Table 3, which were also determined by Dr R. A. Kekwick. The polydispersity of the preparation was approximately determined from the results of the following experiment, which was made on the preparative ultracentrifuge. The sialomucopolysaccharide (2·4 g.) was dissolved to give a 1·8 % (w/v) solution in phosphate buffer (I 0·2, pH 8) which contained 0·15 M-NaCl. The solution was centrifuged at 20 000 ***g*** (average value) for 1 hr. As there was no deposit the ***g*** value was raised first to 40 000, then to 70 000, and each ***g*** force was applied for 1 hr. No deposit was obtained, which indicated that the preparation was substantially free from large-sized molecules. The centrifuge was then run at top speed (105 000 ***g***, average value) for 5 hr., after which time a small pellet of substance was obtained. The contents of the centrifuge tubes were then divided into four approximately equal sections, each about 2 cm.

Table 2. *Intrinsic viscosity η spec./c values of the sialomucopolysaccharide at different concentrations*

c, Concentration measured in g./100 ml.

Concentration (g./100 ml.)	η spec./c
1·00	0·720
0·50	0·656
0·25	0·636
0·125	0·624
0·0625	0·610
Infinite dilution	0·605

Table 3. *Sedimentation constants of sialomucopolysaccharide at various concentrations*

Values corrected to water at 20° (Svedberg & Pederson, 1940).

Concentration (g./100 ml.)	$10^{13} \times S_{20}$
1·10	$4{\cdot}2_7$
0·875	$4{\cdot}9_2$
0·55	$5{\cdot}7_2$

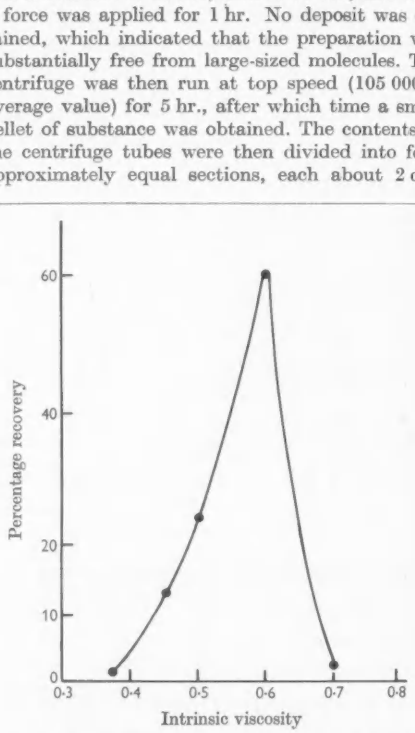

Fig. 2. Plot of intrinsic viscosity [η] of materials, obtained by centrifuging at 105 000 ***g***, against amount of substance in each fraction. Concentration was measured as g./100 ml.

Table 4. *Chemical, physical and serological data*

Analytical values obtained on fractions isolated from the sialomucopolysaccharide after centrifuging at 105 000 ***g*** in phosphate buffer (pH 8) for 5 hr. c, Concentration measured in g./100 ml.

Fraction	Recovery mg.	Recovery %	Sialic acid (%)*	Nitrogen (%)	η spec./c	Le^a activity†
Top	24	1·0	15·2	5·2	0·380	0·025
Second	264	12·4	18·1	4·5	0·455	0·012
Third	520	24·5	17·7	4·6	0·505	0·012
Bottom	1250	60·0	16·8	4·8	0·605	0·025
Sediment	62	2·1	14·0	5·6	0·700	0·012
Original mucopolysaccharide	—	—	17·9	5·0	0·605	0·025

* As *N*-acetylneuraminic acid [Warren's (1959) method].

† Minimum amount (μg.) giving inhibition.

long, starting from the top of the cups, and the solution comprising each layer was carefully removed with a Pasteur pipette, the tip of which was bent upwards. The pellet of substance and the material in each of the sections was recovered after removal of the buffer salts by dialysis. The yields and other analytical data obtained for these fractions are given in Table 4, from which it appears that the bulk of the preparation was composed of molecules with closely similar properties. A plot of the intrinsic-viscosity values for each material against the amount recovered gives a typical Gaussian curve for the molecular-size distribution (Fig. 2), and shows that the majority of the mucopolysaccharide entities are of closely similar size.

Chemical. No precipitate was formed when a 1% (w/v) solution of the material was mixed with an equal volume of the following protein precipitants: trichloroacetic acid (20%, w/v), salicylsulphonic acid (20%, w/v), saturated solutions of tannic acid or picric acid, a 10% solution of lead acetate (in dilute acetic acid), mercuric acetate, aluminium sulphate in 5N-H_2SO_4, phosphotungstic acid, acid or neutral copper sulphate. The analytical figures of the materials obtained as a result of differential high-speed centrifuging (Table 4), indicated that the preparation was remarkably homogeneous in terms of N and sialic acid, and that the latter component was present to the extent of about 18%. The material contained 8·4% of fucose (Table 1), a value considerably lower than that usually found for group-specific A, B and H mucopolysaccharides (about 18%) and somewhat lower than that recorded earlier (about 12%) for preparations of Le^a-specific mucopolysaccharide (Annison & Morgan, 1952).

Table 5. *Amino acid composition of the Le^a specific mucopolysaccharide*

	Amino acid (g./100 g. of mucopolysaccharide)
Aspartic acid	0·49
Threonine	4·48
Serine	1·91
Glutamic acid	0·87
Proline	2·04
Glycine	0·64
Alanine	1·36
Cysteine	0·15
Valine	0·89
Methionine	0·09
Isoleucine	0·38
Leucine	0·87
Tyrosine	0·17
Phenylalanine	0·31
Lysine	0·28
Histidine	0·37
Arginine	0·32

A 1% (w/v) solution of the sialomucopolysaccharide was hydrolysed with 0·5N-HCl for 16 hr. at 100° in sealed ampoules, and the products, which showed considerable humin formation, were assayed for total amino sugar and reducing sugar (Table 1). Analysis of the hydrolysis products by quantitative paper chromatography gave 28·8% of galactose, 20% of glucosamine and 6% of galactosamine, but, owing to extensive humin formation when hydrolysis takes place in the presence of so much sialic acid, these values are subject to a considerably larger error than are those obtained when a sialic acid-free specimen of the mucopolysaccharide is used. Hydrolysis of the material with boiling 6N-HCl and examination of the products on columns of sulphonated polystyrene resins (Moore & Stein, 1951, 1954) was kindly carried out by Dr S. Jacobs of the National Institute for Medical Research. The amino acid composition of the sialic acid-free mucopolysaccharide is given in Table 5, the values obtained being calculated from the ninhydrin values of the effluent fractions. The amino acid composition of the material is similar in pattern to that obtained earlier for other blood-group mucopolysaccharides and shows that the amino acids threonine, serine and proline again make up more than 50% of all the amino acids present, and that the amounts of cysteine, methionine and tyrosine are small. Phenylalanine is present, but in small amounts.

The sialic acid is not released by heating an aqueous solution of the sialomucopolysaccharide at 100°, as reported (Odin, 1956) for the sialic acid-containing cervical mucus or hog seminal gel, but is rapidly eliminated by heating in 0·1N-H_2SO_4 at 80° for 1 hr. or in citrate–HCl buffer at pH 2 and 37° for 24–48 hr.

The sialic acid can also be readily removed from the sialomucopolysaccharide by receptor-destroying enzyme obtained from *Vibrio cholerae*. Sialomucopolysaccharide (0·28 g.) was dissolved in 20 ml. of water. A portion (1 ml.) of the enzyme preparation, which had been purified by adsorption on to and elution from human erythrocytes, was added and the mixture was incubated at 37° for 16 hr. The solution was then dialysed, a thoroughly washed cellophan membrane being used, and the diffusate dried. The indiffusible part was heated at 80° for 10 min., centrifuged to remove any coagulated protein of the receptor-destroying enzyme, and the solution was dried from the frozen state. A single treatment with the enzyme liberated about 30% of the total sialic acid. Repeated treatments, however, as shown in Table 6, removed the sialic acid almost completely. The experimental details and analytical values obtained are given in Table 6.

Immunological. The sialomucopolysaccharide did not inhibit the specific agglutination of A, B or O

Table 6. *Removal of sialic acid from the sialomucopolysaccharide by purified receptor-destroying enzyme*

	Amount of enzyme solution added	Sialic acid content of the sialomuco-polysaccharide (%)	Sialic acid in diffusate (mg.)	Indiffusible material* (mg.)
Original	—	19	—	280
After first treatment	1·0	13	20	236
After second treatment	0·6	10	9·9	198
After third treatment	0·8	3·9	13·1	163
After fourth treatment	0·8	0·2	6·0	124

* Uncorrected for material removed for analysis.

erythrocytes by the appropriate group-specific haemagglutinins, but was a powerful inhibitor of the agglutination of Le(a+) cells by a human anti-Le(a+) serum, and the haemagglutination brought about by the action of three or four completely agglutinating doses of human Le^a antibody on an equal volume of a 1% suspension of Le(a+) red cells in 0·9% sodium chloride solution was completely inhibited by 0·025 μg. of the material. The removal of the sialic acid component by receptor-destroying enzyme or by gentle hydrolysis with acid causes no demonstrable change in the capacity of the material to neutralize anti-Le^a serum (Table 7).

The sialomucopolysaccharide, tested in amounts of 100, 10 and 1 μg. in 0·1 ml. of 0·9% sodium chloride solution, precipitated when mixed with an equal volume of anti-pneumococci type XIV horse serum, as did all other preparations of Le^a substance examined. After removal of the sialic acid component with receptor-destroying enzyme, however, each of the test concentrations gave a considerably increased amount of precipitate when examined under similar conditions.

Virus-inhibitor activity of sialomucopolysaccharide

The knowledge that sialic acid acts as a functional group in those mucosubstances which are powerful inhibitors of viral haemagglutination and the ready elimination of this component with receptor-destroying enzyme, made us determine if the sialomucopolysaccharide possessed any activity as an inhibitor of influenza viruses.

The first experiments were made for us by Professor Wilson Smith and Dr A. Cohen of University College Hospital Medical School, to whom we wish to express our thanks. Three types of influenza-virus strains were used: ASH (type A prime), ROB (type B) and A/Sing/W (Asian type), and the tests were done with both unheated and heated viruses at a constant dose of four agglutinating doses. This ensures the detection of inhibitory activity due to any of the known types of virus-haemagglutination inhibitors. Three classical virus-haemagglutination inhibitors were tested in parallel with the sialomucopolysaccharide, namely, human urinary mucoprotein (Tamm & Horsfall, 1952; Gottschalk, 1958), sheep submaxillary mucoid (McCrea, 1953) and crude egg white.

The results showed that the sialomucopolysaccharide was without appreciable inhibitor activity against unheated ASH and ROB viruses or against A/Sing/W, either heated or unheated. Against heated or 'indicator' ROB virus, however, as little as 0·28 μg. inhibited the haemagglutinating activity of four agglutinating doses of the virus. This result shows that the substance is an extremely potent haemagglutination inhibitor of the α type (Smith, Westwood & Belyavin, 1951), and was similar in this respect to the urinary mucoprotein and the sheep submaxillary mucoid, which had approximately the same degree of activity. The removal of sialic acid from the sialomucopolysaccharide by treatment either with receptor-destroying enzyme or with dilute acid at room temperature resulted in the complete abolition of inhibitory activity (Table 7).

The Le^a activity of the sialomucopolysaccharide is destroyed by an enzyme obtained from *Trichomonas foetus* (Watkins, 1953, and unpublished work). The enzyme, which was largely separated from the neuraminidase (Dr W. M. Watkins unpublished results) present in the crude *T. foetus* extract by zone electrophoresis on a starch column, at pH 6·2 for 24 hr., brought about the destruction of Le^a specificity, whereas the virus-inhibiting capacity of the sialomucopolysaccharide, although decreased owing to the removal of some of the sialic acid, nevertheless remained considerable when tested with the 'indicator' forms of the ASH and ROB strains of influenza virus.

Isolation and identification of N-acetylneuraminic acid

The sialomucopolysaccharide (5 g.) was dissolved in 500 ml. of 0·01 N-H_2SO_4 and adjusted to pH 2 by the addition of 0·1 N-H_2SO_4. The solution was kept at 37° for 24 hr., after which time the acid was neutralized by the addition of $BaCO_3$. The

$BaSO_4$ and excess of $BaCO_3$ were removed and the clear, colourless solution was thoroughly dialysed through a well-washed cellophan membrane against repeated changes of distilled water at 1–2°. The combined diffusates were concentrated to about 30 ml. and treated as a batch operation with Amberlite IR-120 in the hydrogen form to remove cations. The solution was evaporated to dryness, the residue was extracted with methanol (4 ml.) containing a few drops of water, and ethyl ether was added until a slight turbidity developed. After standing overnight at 1–3°, a small precipitate formed and was removed. A few drops of ethyl ether and light petroleum (b.p. range 40–60°) were added to the clear solution to give a faint turbidity and the solution was allowed to stand at 1–3° for several days. A microcrystalline material (80 mg.) separated and was filtered off, washed with a small volume of ice-cold absolute methanol and dried. The mother liquors contained 630 mg. of sialic acid, as determined by Svennerholm's method, and a further amount of microcrystalline material (110 mg.) was later recovered and identified as *N*-acetylneuraminic acid.

A 1 % (w/v) solution of the crystalline material had, after one further recrystallization, a *laevo*-rotation $[\alpha]_D^{18} - 31°$, contained 4·4 % of N and decomposed between 182° and 190°. The authentic *N*-acetylneuraminic acid had $[\alpha]_D^{18} - 32 \pm 2°$, 4·5 % of N and decomposed between 185° and 187°. Under Svennerholm's (1957) conditions the crystalline material gave an extinction (580 mμ) 95·8 % of that given by pure *N*-acetylneuraminic acid. The material behaved on paper chromatograms, with solvent mixtures pyridine–ethyl acetate–acetic acid–water (5:7·5:1:3) or butan-1-ol–propan-1-ol–0·1 N-HCl (1:2:1), as did *N*-acetylneuraminic acid. The crystalline material was converted into pyrrole-α-carboxylic acid, as described by Gottschalk (1953, 1955), by heating in 0·1 N-NaOH at 100° for 15 min., and the resulting product showed a strong absorption maximum at 263 mμ. The material gave no colour on heating with ninhydrin (Moore & Stein, 1954), but an immediate and strong 'direct' colour with a *p*-dimethylaminobenzaldehyde–HCl reagent (Osaki & Turumi, 1947; Hiyama, 1949; Odin, 1952; Gottschalk, 1953). The results indicate that the crystalline material isolated from the sialomucopolysaccharide was *N*-acetylneuraminic acid.

After hydrolysis at pH 2, as described above, the mucopolysaccharide that did not diffuse through cellophan was recovered and its physical, chemical and immunological properties were examined. The material remained electrophoretically homogeneous at pH 8 (Fig. 1*b*), but its mobility, 4·2 cm.^{2} sec.$^{-1}$ v^{-1} × 10^{-5}, was much reduced. The fall in the viscosity found, about 20 %, corresponds closely to that which might be expected from the loss of sialic acid equivalent to about a fifth of the material. The mucopolysaccharide contained 28·8 % of galactose, 22·8 % of glucosamine and 5·8 % of galactosamine. Other analytical figures are given in Table 7. The material showed no loss of Le^a activity, but failed completely to inhibit haemagglutination brought about by the two test strains of heated influenza virus.

Isolation of the Le^a-specific mucopolysaccharide from cyst fluid without the use of phenol

The isolation of the mucopolysaccharide from the cyst fluid by the action of pepsin at pH 1·9, to remove the major part of the co-existing but non-specific protein and thus avoid the use of 90 % phenol, was carried out as follows. The cyst fluid (500 ml.) was mixed with an equal volume of 0·1 M-citrate–HCl buffer at pH 1·9 and digested at 37° with 100 mg. of crystalline pepsin. Toluene was added as a preservative and the digest was frequently shaken during the period of incubation,

Table 7. *Analytical figures of the sialomucopolysaccharide*

Values obtained before and after treatment with (*a*) receptor-destroying enzyme (R.D.E.) and (*b*) acid at pH 2 and 37°.

	Original sialomucopolysaccharide	Mucopolysaccharide (*a*) After R.D.E.	Mucopolysaccharide (*b*) After hydrolysis (pH 2)
Nitrogen (%)	4·8	5·2	5·3
Fucose (%)	8·4	9·1	9·0
Hexosamine (%)*	26·6	29·6	29·7
Reducing sugar (%)*	51·3	55·6	55·0
Sialic acid (%)†	17·9	1·0	0·5
Viscosity (η, in water)	2·88	2·48	2·60
$[\alpha]_D$ in water (*c*, 1 %)	−24°	−24°	−24°
Le^a activity (μg.)‡	0·025	0·025	0·025
Virus activity (μg.)‡	0·28	> 28	—

* See footnote Table 1. † By Warren's (1959) method. ‡ Minimum amount giving inhibition.

and readjusted to pH 1·9 each day with HCl. After several days' incubation the digest was thoroughly dialysed to remove salts, amino acids and low-molecular-weight products of peptic hydrolysis, and the indiffusible material was recovered, dissolved in water to give a 2% solution, saturated with $(NH_4)_2SO_4$ and heated at 60° for about 5 min. A precipitate formed and was collected, thoroughly washed with saturated $(NH_4)_2SO_4$, dissolved in water, the solution was dialysed and the material insoluble in saturated $(NH_4)_2SO_4$ was recovered (10·6 g.). The material soluble in hot saturated $(NH_4)_2SO_4$ was likewise recovered (1·5 g.). A part of the insoluble fraction (5 g.) was dissolved in dimethylsulphoxide and fractionated by the addition of ethanol. The largest fraction (3·3 g.), which separated between 40 and 60% (v/v) of ethanol concentration, was recovered, dissolved in aqueous 2% potassium acetate to yield a 2% solution and fractionated with ethanol. The main fraction (2·5 g.) separated between 45 and 50% (v/v) of ethanol. The analytical figures (Table 8) obtained for this material and for that precipitated between 50 and 55% (v/v) of ethanol give values (Table 7) similar to those obtained for material purified through phenol and treated with acid at pH 2 and 37° to remove *N*-acetylneuraminic acid.

Table 8. *Analytical values of materials isolated from ovarian-cyst fluid by treatment with pepsin*

The dried cyst fluid digested with pepsin at pH 2, saturated with $(NH_4)_2SO_4$ at 60°, fractionated from dimethylsulphoxide by ethanol (40–60%) and subsequently precipitated from aqueous solution by ethanol.

Ethanol fraction (%) ...	45–50 (2·5 g.)	50–55 (0·4 g.)
Nitrogen (%)	5·2	5·5
Fucose (%)	8·2	7·4
Hexosamine (%)*	28·4	30·4
Reducing sugar (%)*	55·0	53·0
Sialic acid (%)†	0·8	0·2
Relative viscosity (η)	3·06	1·62
$[\alpha]_D$ in water (*c*, 1%)	−24°	−26°

* See footnote Table 1.
† Determined by Warren's (1959) method.

Action of papain on sialomucopolysaccharide

There is evidence that the α_1-acid glycoprotein of human plasma is not attacked by the proteolytic enzymes trypsin and papain (Yamashina, 1956) until after the removal of the sialic acid component (10–11%), when digestion readily occurs. It was therefore determined whether the sialomucopolysaccharide, because of its high content of sialic acid, was degraded by papain, it being already known that the blood-group-specific mucopolysaccharides devoid of sialic acid are readily attacked by this enzyme (Pusztai & Morgan, 1958).

Sialomucopolysaccharide (2 g.) was digested with 100 mg. of three-times crystallized papain activated with 2:3-dimercaptopropanol (BAL) at pH 7·6 and 37° for 24 hr., thoroughly dialysed at 1–2° and the indiffusible material was saturated with $(NH_4)_2SO_4$ and warmed to 60°. No precipitate formed, although before digestion with papain the substance was completely precipitated under these conditions. The material was recovered from the saturated $(NH_4)_2SO_4$ solution, thoroughly dialysed to remove salt and dried from the frozen state. A 2% (w/v) solution of the substance was then fractionated by the addition of ethanol containing 2% of potassium acetate and the following ethanol fractions were collected: 50–55% (1·38 g.), 55–60% (0·04 g.), 60–75% (0·25 g.). The number of fractions separating and the yield of each indicates that the material was split largely into two main components and that no appreciable amount of diffusible material was formed during digestion with papain. The analytical results obtained for the products, and for the original sialomucopolysaccharide, are given in Table 9.

Table 9. *Products of the action of crystalline papain on the sialomucopolysaccharide*

Analytical values obtained for materials isolated from sialomucopolysaccharide after digestion with crystalline papain at pH 7·6 and fractionation with ethanol.

Ethanol fraction (%) ...	Original material	Material recovered after digestion with papain		
		0–55	55–60	60–75
Nitrogen (%)	5·0	4·9	4·6	4·7
Fucose (%)	8·4	9·0	8·1	8·8
Hexosamine (%)*	26·5	28·0	24·4	25·2
Reducing sugar (%)*	51·2	53·0	49·2	50·0
Sialic acid (%)†	19·0	18·7	16·6	16·9
Sialic acid (%)‡	17·9	17·9	16·4	16·5
Relative viscosity (η)	2·88	2·10	1·44	1·17
$[\alpha]_D$ in water (*c*, 1%)	−24°	−26°	−20°	−29°
Le^a activity (μg.)§	0·05	0·40	0·80	1·50
Material recovered (%)	—	81·5	2·5	15

*, †, ‡ See footnotes Table 1. § See † footnote, Table 4.

DISCUSSION

A mucopolysaccharide which contains sialic acid as a major carbohydrate component, as well as the fucose, galactose, glucosamine, galactosamine and amino acids which are usually found in the A, B, H and Le^a specific blood-group substances, has been isolated from a single specimen of human ovarian-cyst fluid. The cyst fluid in this instance was obtained from a person of blood phenotype $A_1Le(a+)$, a so-called non-secretor, and, in consequence, showed no group A serological activity but strong and specific Le^a character. Mucopolysaccharides possessing Le^a activity alone have already been obtained, and their immunological properties recorded (Grubb & Morgan, 1949; Annison & Morgan, 1952), but they differ from the Le^a substance now described in that they contain at most only very small and variable amounts of sialic acid, whereas the present material contains about 18% of this acid. In order to differentiate this type of mucopolysaccharide from the closely similar blood-group-specific mucopolysaccharides obtained earlier and to indicate its high content of sialic acid, it has been called sialomucopolysaccharide.

The sialomucopolysaccharide remains insoluble in 90% phenol when the dried ovarian-cyst contents are extracted with this solvent, but dissolves readily in water after phenol extraction and can be further purified by precipitation at 60° from solution in saturated ammonium sulphate. Additional purification is achieved by repeated fractionation from solution in dimethylsulphoxide or water by ethanol. The material so obtained gives clear, viscous 2% solutions in water or 0·9% sodium chloride solution and behaves as an essentially homogeneous substance on the basis of electrophoretic and ultracentrifugal measurements at pH 8. An estimate of the size distribution of the macromolecules in the preparation, based on the amount and intrinsic viscosity of each of the fractions obtained by high-speed centrifuging, indicates that about 85% of the macromolecules have intrinsic viscosity values between 0·50 and 0·60. The average molecular weight of the sialomucopolysaccharide preparation, calculated from the sedimentation constant and the intrinsic viscosity of the whole preparation by means of the formula

$$M = \frac{4690 \times (S_{20,w})^{\frac{1}{2}} \times [\eta]^{\frac{1}{2}}}{(1-\overline{V}\delta)^{\frac{1}{2}}}$$

(cf. Schachman, 1957), where M is the molecular weight, $S_{20,w}$ is the sedimentation coefficient ($S_{20,w}$ 5·72), $[\eta]$ the intrinsic viscosity (0·67), $\overline{V}$ the partial specific volume (0·63) and δ the density of the solution (1·008), gave a value of 237×10^3. This figure is similar to that obtained earlier, 270×10^3 (Kekwick, 1952), for a preparation of Le^a substance which contained about 1% of sialic acid, the diffusion constant for the substance being used in place of intrinsic viscosity.

The removal of sialic acid from the sialomucopolysaccharide by the action of receptor-destroying enzyme lowers its relative viscosity by an amount which corresponds closely to that which might be expected from the lower molecular size after the removal of about 18% of sialic acid. It seems improbable therefore that, in this instance, the sialic acid is itself responsible for the high viscosity of the sialomucopolysaccharide. This conclusion is at variance with that reached by Odin (1958), who considers that the high viscosity of certain ovarian-cyst preparations is due to their content of sialic acid. Similarly, Gibbons (1959) is inclined to believe that since the sialic acid of oestrous and pregnancy specimens of bovine cervical mucins is the only major chemical distinction between them, it therefore plays a part in determining the degree of molecular expansion which, in turn, is responsible for the consistency of the mucus.

Electrophoretic examination at pH 8 gives the mobility of the sialomucopolysaccharide as $-7{\cdot}6$ cm.^{2} v^{-1} sec.$^{-1} \times 10^{-5}$, which is much greater than the mobility, $-4{\cdot}2$ cm.^{2} v^{-1} sec.$^{-1} \times 10^{-5}$, of the mucopolysaccharide obtained from it by removing the sialic acid enzymically with receptor-destroying enzyme. The true mobility value for the mucopolysaccharide could be somewhat lower than this figure as, after treatment with the enzyme, the material examined still retained about 1% of sialic acid.

The sialic acid was readily split from the sialomucopolysaccharide by treatment with sulphuric acid at pH 2 and 37° and was identified as *N*-acetylneuraminic acid. Although it is probable that *N*-acetylneuraminic acid is present in the sialomucopolysaccharide, one cannot yet be completely certain of this, as it is well known that the *O*-acetyl groups in the *ON*-diacetylneuraminic acids (Blix, 1959) are extremely labile and are frequently lost during the isolation and purification of sialic acid-containing materials. However a sialic acid other than *N*-acetylneuraminic acid does not yet appear to have been described in materials derived from human sources.

It has long been known (cf. Burnet, 1951) that various strains of influenza virus and other closely related viruses, such as those of mumps and Newcastle disease in fowls, agglutinate vertebrate erythrocytes and that the haemagglutination can be inhibited by many different muco-substances (Anderson, Burnet, Fazekas de St Groth, McCrea & Stone, 1948). Recent work has shown that highly purified mucoprotein obtained from urine and from submaxillary glands, the acid α_1 and α_2 muco-

proteins of serum and the ovomucin fraction of egg white are very powerful inhibitors and, on the basis of many earlier observations of Gottschalk (cf. Gottschalk, 1956), Odin (1952) and Klenk & Laüenstein (1952), first identified the essential functional group in these different mucoproteins as sialic acid.

The sialomucopolysaccharide also inhibits the haemagglutination of fowl erythrocytes by the heated or 'indicator' form of two strains of influenza virus, ASH (type A prime) and ROB (type B), and has about the same capacity to inhibit haemagglutination brought about by these viruses as have the highly purified preparations of human urinary mucopolysaccharide and sheep submaxillary mucoid included in the assay. Neither the unheated nor the heated form of an Asian type of influenza virus was inhibited by the sialomucopolysaccharide. The removal of sialic acid from the sialomucopolysaccharide by receptor-destroying enzyme or by incubation at 37° with sulphuric acid at pH 2 gives a mucopolysaccharide which does not inhibit haemagglutination brought about by heated virus.

The sialomucopolysaccharide, or the mucopolysaccharide derived from it after the removal of sialic acid by acid or by receptor-destroying enzyme, are potent inhibitors of Le^a-specific isoagglutination. The ready removal of sialic acid from sialomucopolysaccharide by purified enzyme without the release of other components, and the recovery of a mucopolysaccharide containing all the sugars originally present except sialic acid, indicates that sialic acid units are most probably attached as single end residues or as side units and are not built into the main carbohydrate chains. In any event, it is clear that they in no way demonstrably interfere with the reactivity of the blood-group Le^a-specific carbohydrate structures which are also considered to be present as non-reducing end groups in the Le^a-active mucopolysaccharides (Watkins & Morgan, 1957). The sialomucopolysaccharide obtained after the Le^a-specific structures have been destroyed by an enzyme obtained from *T. foetus* is still active as an inhibitor of virus haemagglutination and largely retains its sialic acid component.

The complete independence of each of the biologically active structures in the sialomucopolysaccharide is emphasized by the ready destruction of each specific character by the appropriate enzyme, with the result that, from a sialomucopolysaccharide which has both activities, a mucopolysaccharide which possesses either Le^a specificity or anti-viral activity alone can be obtained.

The sialomucopolysaccharide precipitates with anti-pneumococci type XIV horse serum, as was observed earlier (Annison & Morgan, 1952) for an Le^a mucopolysaccharide which contained no appreciable amount of sialic acid. This cross-reactivity is considerably enhanced when the *N*-acetylneuraminic acid is removed by receptor-destroying enzyme, so that the removal of sialic acid brings about the exposure of structures which react with type XIV antibody just as does the removal of fucose (cf. Kabat, 1956).

The isolation of a mucopolysaccharide free from sialic acid when the Le^a substance was recovered from the crude ovarian-cyst fluid, by incubation with pepsin at pH 1·9 to remove the co-existing protein, is not surprising in view of the rapidity with which this component is removed from the sialomucopolysaccharide, and indeed from all sialic acid-containing mucoproteins, by dilute acid. It is evident that naturally occurring sialomucopolysaccharides cannot be obtained by any procedure which involves acid digestion with pepsin, a method so frequently used to recover the blood-group-specific substances (cf. Kabat, 1956). On the other hand, the recovery of sialomucopolysaccharide by the extraction of the dried crude ovarian-cyst fluid with 90% (w/w) phenol is readily achieved with no demonstrable loss of the sialic acid component. The further purification of the sialomucopolysaccharide by treatment with saturated ammonium sulphate at 60°, to remove similar material of smaller molecular weight and other impurities, likewise does not eliminate the sialic acid component.

In the light of considerable experience with the use of 90% phenol rather than peptic digestion for the removal of contaminating protein for the isolation of the blood-group-specific mucopolysaccharides, it is evident that most native mucopolysaccharides obtained from pseudomucinous cyst fluids contain only a small amount of sialic acid. On the evidence at present available it is not known if the specific substance is secreted as a sialomucopolysaccharide, which then loses all or part of its sialic acid component by the action of tissue neuraminidases during the time needed for the mucopolysaccharide to accumulate *in vivo*, or is in most instances secreted as a mucopolysaccharide which is free from sialic acid. It is observed, however, that many of the mucopolysaccharide preparations recovered by phenol extraction contain small and variable amounts of sialic acid, the presence of which could be considered as the survival of a small part only of an original major component of sialic acid, or the failure of the synthetic step which adds sialic acid. The contamination of a mucopolysaccharide that is free from sialic acid by a sialomucopolysaccharide similar to that described in this paper, would be readily detected by electrophoretic examination.

The changes induced in the sialomucopolysaccharide by papain are similar to those reported earlier for the blood-group-specific mucopolysac-

charides which were devoid of a major sialic acid component, and it appears therefore that the presence of a considerable amount of *N*-acetylneuraminic acid in the macromolecule does not prevent the degradation of the sialomucopolysaccharide by this proteolytic enzyme.

SUMMARY

1. A sialomucopolysaccharide isolated from ovarian-cyst fluid obtained from a person of blood phenotype A Le(a+) is closely similar in chemical composition and physical properties to the blood-group-specific mucopolysaccharides obtained earlier from pseudomucinous ovarian-cyst fluids. The material contains 18% of sialic acid in addition to the fucose, galactose, glucosamine, galactosamine and amino acids usually found.

2. The sialic acid, which is most probably present as *N*-acetylneuraminic acid, is readily removed by acid at pH 1·9 and 37° or by receptor-destroying enzyme.

3. The sialomucopolysaccharide is an active Le^a-specific substance and a potent inhibitor of the haemagglutination by heated type A and B influenza viruses. The removal of the sialic acid component by receptor-destroying enzyme leaves a mucopolysaccharide which has no capacity to inhibit viral haemagglutination, but is fully active as a Le^a substance.

4. The destruction of the Le^a specificity by a Le^a-destroying enzyme obtained from *Trichomonas foetus* leaves a mucopolysaccharide which contains sialic acid and is an inhibitor of viral haemagglutination.

5. The sialomucopolysaccharide precipitates with anti-pneumococci Type XIV horse serum. The degree of precipitation is considerably enhanced after the removal of the sialic acid by receptor-destroying enzyme.

6. The sialomucopolysaccharide is an example of a naturally occurring macromolecule which possesses two biological activities, each of which is associated with a different chemical structure.

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The Enzymic Formation of Haem by Human and Rat Tissues

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Previous studies have shown that chicken erythrocytes and human reticulocytes contain iron-incorporating enzyme or enzymes, which incorporate iron into protoporphyrin to form haem (Goldberg, 1959). In the adult, the tissues predominantly concerned in haem synthesis are the bone marrow and liver. In the foetus, and in pathological states of extramedullary erythropoiesis in the adult, the liver and spleen play a major role in haem synthesis. Gibson, Neuberger & Scott (1955) have shown that the enzyme δ-aminolaevulic dehydrase is present not only in bone marrow and liver, but also in the kidney and spleen of the rabbit. The experiments described in this paper were undertaken to determine the distribution and activities of the iron-incorporating enzyme in human and rat tissues.

Bessis & Breton-Gorius (1957) have shown by electron microscopy that iron is preferentially located in the mitochondria of the haemoglobin-forming erythroblast. These histological studies have been a stimulus to determine which intracellular component is the richest source of iron-incorporating enzyme. Ascorbic acid and reduced glutathione potentiate the enzyme system when it is derived from chicken blood. Further investigations were therefore carried out to determine the effect of these substances on the iron-incorporating enzyme derived from human and rat tissues.

MATERIALS

Radio iron ($^{59}FeCl_3$; specific activity 1–3 mc/mg. of iron) was obtained from A.E.R.E., Harwell. Protoporphyrin methyl ester, prepared from blood by the method of Grinstein (1947), was a gift from Professor C. Rimington, F.R.S. The ester was hydrolysed with 25% HCl for 16 hr., the free protoporphyrin was dried in a desiccator, suspended in 0·142 M-sodium bicarbonate and the final solution adjusted to pH 7·4. Ascorbic acid and reduced glutathione (GSH; British Drug Houses Ltd.) were dissolved in water and the resultant solutions were adjusted to pH 7·4 before use. The water used throughout the experiments was purified by passage through an Elgastat deionizer. The glassware was washed with chromic acid, then with 2 N-HCl and finally with water.

METHODS

Rat tissues. Young female albino rats, Wistar strain, were anaesthetized with ether and killed by exsanguination. Rat intestine was washed free of its contents by repeated washes with 0·9% sodium chloride solution. Each tissue was washed with water, dried on filter paper, weighed and homogenized with three or seven times its volume of 0·25 M-sucrose in an ice-cooled Potter glass homogenizer. To the homogenate was added one-quarter of its volume of 0·45 M-2-amino-2-hydroxymethylpropane-1:3-diol (tris) buffer, pH 7·4. With liver homogenates, intracellular constituents were fractionated (from a 1:8 homogenate), by the method of Schneider (1948), into nuclei, mitochondria and microsomes plus cell sap. Each fraction was suspended in 0·25 M-sucrose to the same volume as the original homogenate, and 0·25 vol. of 0·45 M-tris buffer, pH 7·4, was added.

Human tissues. Samples of spleen and kidney removed from patients undergoing surgery for splenectomy or nephrectomy were treated similarly. Biopsies of normal liver were obtained from patients undergoing upper abdominal surgery. Bone-marrow samples were obtained in the following way: a 10 ml. sample of mixed bone marrow and blood was withdrawn from the iliac crest by the method of Rheingold, Weifuse & Dameshek (1949) with a heparin-

ized syringe and transferred to an ice-cooled heparinized measuring cylinder. The blood–bone-marrow tissue was haemolysed by addition of 20 ml. of water and iso-osmotic concentration was restored with 5 ml. of 0·45 M-tris, pH 7·4. The incubation was performed in 50 ml. Erlenmeyer flasks, each of which contained a portion of the material whose enzyme activity was to be measured (in 1–5 ml.), 20 μM-protoporphyrin, 1 μC of $^{59}FeCl_3$, 1 ml. 0·45 M-tris, pH 7·4, 2 mg. each of penicillin G and streptomycin sulphate, to prevent bacterial contamination, and 0·9% sodium chloride solution to 10 ml. Ascorbic acid and GSH when used were each contained in a volume of 1 ml. Ascorbic acid was L-ascorbic acid, unless otherwise stated. The pH of the flask contents rose during the incubation by about 0·2–0·3 pH unit. After incubation at 37° for 3 hr. with mechanical shaking (90 shakes/min.) the reaction was stopped by addition of 1 ml. of M-sodium cyanide.

Carrier haemoglobin (25 ml.) from human blood was added to each flask to facilitate isolation of haem (Goldberg, Ashenbrucker, Cartwright & Wintrobe, 1956). This carrier haemoglobin solution was prepared by haemolysing 1 vol. of washed human red cells with 1 vol. of water. Iso-osmotic concentration was restored by addition of sodium chloride to a concentration of 0·9% (w/w). The flask contents were homogenized for 3 min. in a Waring Blendor and centrifuged at 1600 *g* for 20 min. to remove tissue debris. Haem was isolated from the supernatant fluid by the method of Fischer (1941) and purified and recrystallized by the method of Shemin, London & Rittenberg (1950). Weighed samples of the isolated haem were counted in a well-type scintillation counter with a counting efficiency for iron of 22%. The standard error of the counting procedure was ±2% and the reproducibility of the system as a whole was ±5%.

Table 1. *Ascorbic acid and reduced glutathione concentrations in rat-liver homogenate before and after dialysis against water at 4° for 22 hr.*

Ascorbic acid (μg./g. of tissue)		GSH (μg./g. of tissue)	
Before dialysis	After dialysis	Before dialysis	After dialysis
161	8	2200	400
177	6	2200	600
159	4	1800	300

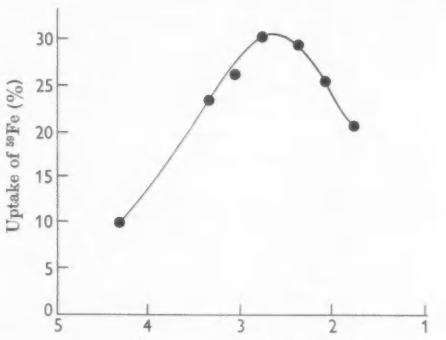

Fig. 1. Effect of concentration of ascorbic acid on the iron-incorporating-enzyme activity of dialysed rat-liver homogenate at pH 7·4.

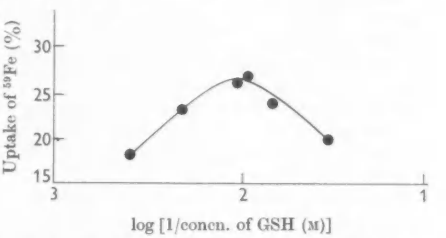

Fig. 2. Effect of varying concentration of reduced glutathione on the iron-incorporating-enzyme activity of dialysed rat-liver homogenate at pH 7·4.

Table 2. *Comparison of iron-incorporating-enzyme activities of intracellular constituents of rat-liver homogenate in sucrose and the effect of ascorbic acid and reduced glutathione on these activities*

Incubations were carried out under standard conditions at pH 7·4 (see Methods section) with 1 μC of $^{59}FeCl_3$ and 20 μM-protoporphyrin, with and without the addition of ascorbic acid (2 mM) or GSH (15 mM). Control flasks were made up in the same way as the test flasks but contained no enzyme source.

Expt. no.	Tissue source	Uptake of ^{59}Fe/g. of wet tissue (%)		
		No addition	With ascorbic acid	With GSH
1	Homogenate	6·3	8·7	8·4
	Nuclei	0·6	1·2	0·6
	Mitochondria	4·8	12·7	6·6
	Microsomes and cell sap	0·9	3·3	1·8
	Control	<0·1	0·4	<0·1
2	Homogenate	3·5	3·8	4·0
	Nuclei	0·5	2·2	1·9
	Mitochondria	2·5	6·2	4·2
	Microsomes and cell sap	0·3	1·8	1·5
	Control	—	0·2	<0·1

Ascorbic acid and GSH contents of the homogenate were determined by the methods of Roe & Kuether (1943) and Woodward & Fry (1932) respectively. Haemoglobin concentration was measured by the alkaline-haematin method of Clegg & King (1942).

RESULTS

All results are expressed as the percentage of added radio iron which was incorporated into the haem. The total number of counts in the haem was obtained by multiplying the counts per milligram of haem by the weight of haem (mg.) in each flask after the addition of the carrier haemoglobin. In the absence of added protoporphyrin there was no significant incorporation of iron into haem by any tissue system.

Rat tissues

Liver. Ten rat-liver homogenates gave an average uptake of ^{59}Fe of 4·8 %/g. wet wt. of tissue (S.D. ± 2·4, range 1·2–9·3 %). Addition of ascorbic acid and GSH stimulated iron incorporation into protoporphyrin. After dialysis against water at 4° for 24 hr., the enzymic activity of the homogenate was decreased to about 15 % of the original value, but addition of ascorbic acid or GSH restored the dialysate to its former activity. The GSH and ascorbic acid contents of three liver homogenates before and after dialysis are shown in Table 1. The effects of varying concentrations of ascorbic acid and reduced glutathione on dialysed rat-liver preparations are shown in Figs. 1 and 2 respectively. Maximum potentiation occurred at about 2 mM-ascorbic acid and 15 mM-GSH.

The enzyme activities of the intracellular constituents of liver isolated from a sucrose homogenate are shown in Table 2. Most of the activity is present in the mitochondrial fraction. Ascorbic acid and GSH increased the activity of all intracellular constituents.

Kidney and spleen. Kidney homogenates showed an average uptake of ^{59}Fe of 0·8 %/g. wet wt. in eight samples tested (S.D. ± 0·4, range 0·2–1·2 %) and spleen homogenates gave a similar incorporation with a mean value of 0·6 % in six samples (S.D. ± 0·3, range 0·3–1 %). Another tissue that showed marked enzyme activity was heart muscle, which had an average uptake of 1·6 % (S.D. ± 0·3 %) in six samples. Small intestine, brain and leg muscle had very little enzymic activity (0·1 % incorporation). Heat-treatment, i.e. placing in a boiling-water bath for 4 min., caused almost complete loss of activity in each of the tissue homogenates.

Human tissues

Bone marrow. Bone marrow showed small but significant iron-incorporating-enzyme activity in nine samples tested (Table 3). Normal human

Table 3. *Iron-incorporating-enzyme activity of iliac-crest 'bone marrow–blood' tissue in normal and pathological states and the effect of ascorbic acid and reduced glutathione on this activity*

Standard incubation procedure was followed with 1 μC of $^{59}FeCl_3$ and 20 μM-protoporphyrin with the addition where stated of 1·4 mM-ascorbic acid or 6·5 mM-GSH. Samples of the blood–bone-marrow tissue were analysed chemically for ascorbic acid, GSH and iron. Hb, Haemoglobin; P.C.V., packed-cell volume.

Patient no.	Diagnosis	Age (years)	Sex	Hb (g./100 ml.)	P.C.V. (%)	Uptake of ^{59}Fe/g. of wet tissue (%)			Marrow-blood analysis		
						Marrow alone	Marrow + ascorbic acid (1·4 mM)	Marrow + GSH (6·5 mM)	Ascorbic acid (mg./100 ml.)	GSH (mg./100 ml.)	Iron (μg./100 ml.)
1	Osteoarthritis	57	M	15·9	49·5	0·3	0·7	0·7	1·8	14	—
2	Rickets	14	M	13·0	44·5	0·5	0·5	0·5	1·2	23	108
3	Thyrotoxicosis	51	M	14·4	47·0	0·2	0·5	0·6	2·5	13	—
4	Cerebral thrombosis	57	M	15·9	47·5	0·2	0·2	—	1·2	—	83
5	Combined deficiency of vitamin B_{12} and folic acid	54	M	10·7	34·5	0·3	0·5	0·5	2·1	22	—
6	Bronchial carcinoma; deposits in bone marrow	62	F	13·9	42·5	0·3	1·4	1·5	0·5	6	—
7	Scurvy (treated)	58	M	8·4	25·0	0·3	0·4	0·3	0·97	19	100
8	Haemochromatosis	57	M	13·6	43·0	0·4	0·3	0·5	1·6	16	164
9	Thyrotoxicosis; iron-deficiency anaemia	59	F	11·1	39·0	0·1	0·3	—	0·96	—	70

blood does not have significant iron-incorporating activity (Goldberg, 1959). In only one case (no. 6) did the addition of ascorbic acid or glutathione significantly increase the activity of the bone-marrow samples. In this case (a patient with bronchial carcinoma and bone-marrow metastases) the marrow ascorbic acid and glutathione concentrations were very low. The marrow from a patient with treated scurvy and a patient with haemochromatosis showed values that were within the normal range.

Liver. A sucrose homogenate of liver contained significant iron-incorporating-enzyme activity, most of which was present in the mitochondria (Table 4). The addition of ascorbic acid (0·14 mM) and GSH (6·5 mM) potentiated the activity of all the cellular constituents, whereas heat-treatment caused complete loss of activity of the homogenate alone and of the homogenate to which ascorbic acid (0·14 mM) and GSH (6·5 mM) had been added.

Spleen. Iron-incorporating activity was found in homogenates of two human spleens (Table 5) removed from patients with acquired haemolytic anaemia and myelofibrosis respectively. Dialysis of the homogenates against water for 24 hr. decreased the activities to 10% and 22% respectively. Addition of ascorbic acid or GSH greatly potentiated the activity of the dialysate in both cases. There was histological evidence of extramedullary erythropoiesis in the spleen from the patient with myelofibrosis, but not in the spleen from the patient with acquired haemolytic anaemia. The enzymic activity per unit weight of the former was about four times that of the latter.

Table 4. *Comparison of the iron-incorporating-enzyme activities of intracellular constituents of human-liver homogenate in sucrose and the effect of ascorbic acid and reduced glutathione on these activities*

Standard procedure for incubation was followed at pH 7·4 with 1 μc of $^{59}FeCl_3$ and 20 μM-protoporphyrin. Ascorbic acid and GSH were added as indicated. Control flasks contained 0·9% sodium chloride solution in place of a source of enzyme.

Expt. no.	Tissue source	Uptake of ^{59}Fe/g. of wet tissue (%): No addition	With L-ascorbic acid (0·14 mM)	With GSH (6·5 mM)
1	Homogenate	1·7	2·3*	44·4
	No enzyme source	<0·1	—	<0·1
2	Homogenate	1·2	3·5	10·9
	Mitochondria	1·0	1·0	2·8
	No enzyme source	<0·1	<0·1	—
3	Homogenate	3·4	6·8	15·3
	Nuclei	0·1	—	—
	Mitochondria	2·6	3·0	4·0
	Microsomes and cell sap	0·3	—	—
	No enzyme source	<0·1	<0·1	—

* D-Ascorbic acid gave similar incorporation.

Table 5. *Iron-incorporating-enzyme activities of samples of spleen from a case of haemolytic anaemia and from a case of myelofibrosis and of samples from two kidneys; effect on the activity of these samples of dialysis and of the addition of ascorbic acid and reduced glutathione to dialysed and undialysed samples*

Tissue samples were homogenized in sucrose and incubated with 1 μc of $^{59}FeCl_3$ and 20 μM-protoporphyrin under standard conditions. Control flasks containing no enzyme source gave an uptake of ^{59}Fe into haem of less than 0·1%.

Case	Tissue	Uptake of ^{59}Fe/g. of wet tissue (%): No addition	With ascorbic acid (0·14 mM)	With GSH (6·5 mM)
Haemolytic anaemia	Spleen homogenate	1·3	1·8	2·7
	Dialysed spleen homogenate	0·1	2·0	1·2
Myelofibrosis	Spleen homogenate	5·4	12·8	15·1
	Dialysed spleen homogenate	1·2	19·6	31·1
Nephrectomy (renal calculus)	Kidney homogenate	0·7	5·7	42·2
Nephrectomy (renal neoplasm)	Kidney homogenate*	1·3	4·0	12·8

* Sample obtained from non-tumour-bearing area.

Kidney. Two samples of kidney showed iron-incorporating-enzyme activities of 0·7% and 1·3% respectively. The addition of ascorbic acid and GSH potentiated these activities (Table 5).

DISCUSSION

These experiments have demonstrated the presence of the iron-incorporating enzyme in human and rat liver, spleen and kidney and in rat-heart muscle as well as in human bone marrow. The enzyme activity is present in human tissues that synthesize haemoglobin in adult or foetal life or in pathological states of extramedullary erythropoiesis; the spleen in which this was present contained four times the enzyme activity per unit weight of that in which extramedullary erythropoiesis was absent. The distribution of the iron-incorporating enzyme in human and rat tissues is similar to the distribution of δ-aminolaevulic dehydrase. Thus Gibson, Neuberger & Scott (1955) found the latter enzyme in the bone marrow, liver, kidney and spleen of the rabbit.

Fractionation of the cellular contents of human and rat liver demonstrated that the mitochondria were the most active source of the iron-incorporating enzyme. This finding is important in view of the work of Bessis & Breton-Gorius (1957), who showed that ferritin iron is mainly concentrated in the mitochondria of the erythroblast. Minikami (1958), using techniques substantially different from those used by us, was able to show the presence of the iron-incorporating enzyme in rat liver, kidney, spleen, heart, brain and muscle and in rat-liver mitochondria. On the other hand, Rabinowitz & Olsen (1958) have suggested that in rabbit reticulocytes the microsomes are responsible for incorporation of iron into haemoglobin.

Goldberg (1959) demonstrated that ascorbic acid and GSH in concentrations of the order present in human or chicken blood potentiate the iron-incorporating enzyme when it is derived from chicken blood. It has now been shown that these physiological reducing substances potentiate incorporation of iron in several human and rat tissues. Dialysis of tissue homogenates not only caused a marked reduction of their ascorbic acid and GSH contents (Table 1) but also significantly decreased their enzymic activities, whereas addition of ascorbic acid and GSH to the dialysed homogenates often more than restored the activities. Thus the initial loss of activity was directly related to the loss of reducing substances. Mazur, Baez & Shorr (1955) have suggested that ascorbic acid and GSH mobilize ferritin iron for haemoglobin synthesis and the present study has certainly confirmed the important bearing of ascorbic acid and GSH on iron incorporation.

SUMMARY

1. An enzyme system that incorporates iron into protoporphyrin has been demonstrated in human and rat liver, kidney and spleen and in human bone marrow. Studies on the intracellular components of human and rat liver showed that whereas iron incorporation occurred with all fractions, those containing the mitochondria were most active.

2. Ascorbic acid and reduced glutathione potentiated the iron-incorporating-enzyme activity of all tissues. Dialysis of tissue homogenates led to loss of activity which was restored by addition of ascorbic acid or glutathione. The results confirm the important role which these substances play in the incorporation of iron.

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Studies on Glucosaminidase

4. THE FLUORIMETRIC ASSAY OF *N*-ACETYL-β-GLUCOSAMINIDASE*

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Mead, Smith & Williams (1955) introduced a method for the fluorimetric assay of β-glucuronidase activity; the method consisted of the enzymic hydrolysis of methylumbelliferyl β-glucuronide, followed by fluorimetric estimation of the liberated methylumbelliferone. Robinson (1956) synthesized methylumbelliferyl β-glucoside and used it in the study of β-glucosidases from a variety of sources; like Mead *et al.* (1955), Robinson found the fluorogenic method convenient and extremely sensitive. Levvy, Hay & Marsh (1957) attempted to use methylumbelliferyl β-glucuronide in the study of limpet β-glucuronidase; they found methylumbelliferone to be unstable, and so considered the method to be too exacting for general use.

In the present paper, a preparation of *N*-acetyl-β-glucosaminidase from ram testis has been used to evaluate the fluorogenic methylumbelliferyl *N*-acetyl-β-glucosaminide as a substrate. A preliminary account of this work has appeared (Leaback & Walker, 1960).

MATERIALS AND METHODS

Methylumbelliferone. Calibration curves were constructed with 4-methylumbelliferone (British Drug Houses Ltd.) purified (m.p. 186–187°) according to Mead *et al.* (1955).

Methylumbelliferyl N-acetyl-β-glucosaminide. Methylumbelliferone (1·9 g.) and 5 g. of acetochloroglucosamine (Leaback & Walker, 1957) were dissolved in cold acetone (70 ml.) and 16·5 ml. of N-NaOH was added. After standing for 6 hr. at room temperature, followed by several days at 5°, the acetylated glycoside crystallized; only a small amount remained in solution. The solid was recrystallized from ethanol to give 2·9 g. (37% yield) of methylumbelliferyl tetra-acetyl-β-glucosaminide (4-methyl-2-oxo-1:2-benzopyran-7-yl-2′-acetamido-2′-deoxy-3′:4′:6′-tri-*O*-acetyl-β-D-glucopyranoside), m.p. 254–255°; $[\alpha]_D^{20}$ −18·9° in $CHCl_3$ (*c*, 1), (Found: C, 56·8; H, 5·5; N, 2·9. $C_{24}H_{27}O_{11}N$ requires C, 57·0; H, 5·6; N, 2·8%).

The acetylated glycoside (1 g.) was suspended in 50 ml. of anhydrous methanol and on the addition of 1 ml. of N-sodium methoxide the compound went rapidly into solution; after a few minutes, the de-*O*-acetylated compound started crystallizing. The mixture was kept overnight, and the solid filtered off. The solid was recrystallized once from hot water, and traces of methylumbelliferone were removed by stirring (magnetically) a suspension of the product in acetone (100 ml.) for 16 hr.; filtration gave 0·49 g. (62% yield) of methylumbelliferyl *N*-acetyl-β glucosaminide (4-methyl-2-oxo-1:2-benzopyran-7-yl-2′-acetamido-2′-deoxy-β-D-glucopyranoside), m.p. 212–214°; $[\alpha]_D^{20}$ −14·5 in H_2O (*c*, 0·1), (Found: C, 57·1; H, 5·6; N, 3·6. $C_{18}H_{21}O_8N$ requires C, 57·0; H, 5·5; N, 3·7%).

Tests were carried out for the presence of free methylumbelliferone in solutions of the glycoside. With the optical system described below, the fluorescence of a solution of the glycoside (66 μM) was the same at pH 7 and pH 10·3. Mead *et al.* (1955) showed that the fluorescence of methylumbelliferone increased greatly with pH over this range. It was estimated that the presence of 0·002% of free methylumbelliferone would have been detectable by this test. The absence of free methylumbelliferone in fresh solutions of the glycoside was confirmed by chromatography on Whatman no. 1 paper, by using downward elution with the non-polar phase of butanol–ethanol–water (4:1:5, by vol.). Spots were rendered visible beneath a mercury lamp (Wood's-glass filter) by heating the chromatogram briefly to 105° and exposing it to ammonia vapour. Methylumbelliferyl *N*-acetyl-β-glucosaminide and (if present) methylumbelliferone showed as fluorescent spots of R_F 0·6 and 0·9 respectively.

Spectra. Fluorescence and excitation spectra were determined with an Aminco–Bowman spectrofluorimeter; the instrument was standardized with mercury emission lines and by the method of Sprince & Radley (1956). The ordinates of the spectra (Figs. 1, 2) are arbitrary (dependent in part upon the characteristics of the instrument), but the 'mirror-image' character (see Bowen & Wokes, 1953) of the excitation and fluorescence peaks suggested that the curves were not seriously at fault. For the sake of clarity, the spectra in Figs. 1 and 2 were discontinued short of small scatter peaks.

Absorption spectra were measured in a Unicam SP. 500 spectrophotometer. The small difference in wavelength between the absorption and excitation maxima might be due to the characteristics of the spectrofluorimeter, or to uncertainties in the measurement of absorption spectra of fluorescent compounds (cf. White, Hoffman & Magee, 1957).

Estimation of methylumbelliferone

Spectrophotometric assay. Methylumbelliferone was estimated in alkaline solution with a Unicam SP. 500 spectrophotometer. The absorption at 360 mμ of solution in

* Part 3: Woollen, Heyworth & Walker (1961).

0·15 M-glycine buffer pH 10·3 was linear with concentration over the range (I) 0·5–11 μg. of methylumbelliferone/ml.

Fluorimetric assay. Fluorimetric estimations of methylumbelliferone were carried out on a Locarte fluorimeter (see Laurence, 1957). The instrument was fitted with quartz optics and a 1 ml. cuvette holder. On the primary side, a Corning 5860 filter together with a Barr and Stroud 365 mμ interference filter were used: the secondary-side filter was a Corning 3389.

In 0·15 M-glycine buffer, pH 10·3, linear calibration curves were obtained for methylumbelliferone concentrations of 0·01–0·45 μg./ml. (range II) and 0·0025–0·10 μg./ml. (range III) when read against a standard solution of quinine bisulphate (2 μg./ml. in 0·1 N-sulphuric acid).

The fluorescence of methylumbelliferone was unaltered in the presence of the enzyme preparation, albumin, citrate buffer and the substrate, at concentrations used in reaction mixtures. It was not necessary to take special precautions to exclude dust from the solutions.

Preparation and assay of enzyme

At high dilutions, the partially purified preparation from ram testis (Borooah, Leaback & Walker, 1961) showed erratic enzyme activity. J. W. Woollen (personal communication) overcame similar difficulties by the addition of 0·01% of crystalline bovine-serum albumin (Armour Products) to the reaction mixture; after the adoption of this procedure, no further difficulties of this kind were experienced. Unless otherwise stated, the enzyme was incubated at 37° for 30 min. with 0·05 M-sodium citrate buffer pH 5·0, 0·01% albumin and 0·264 mM-methylumbelliferyl *N*-acetyl-β-glucosaminide; appropriate blanks were also incubated. The enzyme reaction was stopped, and liberated methylumbelliferone was converted into the anionic form by the addition of 3 ml. of 0·2 M-sodium glycinate buffer, pH 10·65. Methylumbelliferone was then estimated spectrophotometrically (range I) or fluorimetrically (range II or III) according to the amount present.

The addition of 3 ml. of 0·2 M-glycine buffer, pH 10·65 to reaction mixtures (1 ml.) buffered with 0·05 M-citrate to values between pH 3·78 and pH 6·0, gave solutions of pH 10·2–10·6. No change in the fluorescence of methylumbelliferone was detectable within this range, so that adjustment of the final pH before estimation was not necessary (cf. Levvy *et al.* 1957).

RESULTS

Spectra. The excitation and fluorescence spectra of methylumbelliferone (Fig. 1) and methylumbelliferyl *N*-acetyl-β-glucosaminide (Fig. 2) were determined in 0·15 M-glycine buffer, pH 10·3. It can be seen that both compounds fluoresce strongly, and that the excitation maximum of the methylumbelliferone anion falls close to the strong mercury emission line at 365 mμ. The fluorimetric estimation of methylumbelliferone in the presence of the glycoside therefore required a filter system which transmitted only the 365 mμ line on the primary side, and wavelengths above 405 mμ on the secondary side. The system chosen was satisfactory since the blanks due to scatter and to the fluorescence of the glycoside (0·066 mM) and glycine buffer (0·15 M) were equivalent to about 0·0015 μg. of methylumbelliferone/ml. As Levvy *et al.* (1957) found, the glycine buffer itself showed a small but significant fluorescent blank; this blank was unaffected by recrystallizing the glycine and was of the expected order for the Raman effects described by Parker (1959).

Fig. 3 shows the absorption spectra of methylumbelliferone ($\lambda_{max.}$ 360 mμ, ϵ 16 720) and the *N*-acetyl-β-glucosaminide ($\lambda_{max.}$ 317 mμ, ϵ 13 620) in glycine buffer, pH 10·3; the absorption of the glycoside resembles that of methylumbelliferone in neutral solution ($\lambda_{max.}$ 320 mμ, ϵ 12 800). Thus, in

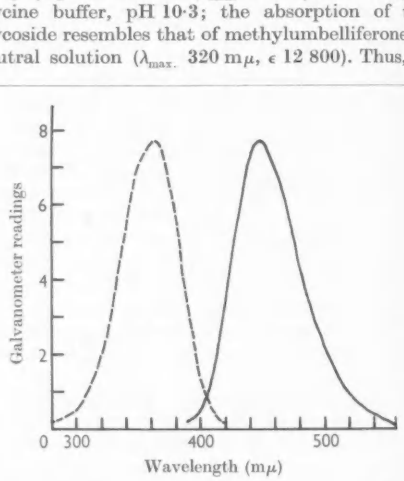

Fig. 1. Fluorescence and excitation spectra of methylumbelliferone (0·1 μg./ml.) in 0·15 M-glycine buffer, pH 10·3. ——, Fluorescence spectrum, $\lambda_{max.}$ 448 mμ; - - -, excitation spectrum, $\lambda_{max.}$ 364 mμ.

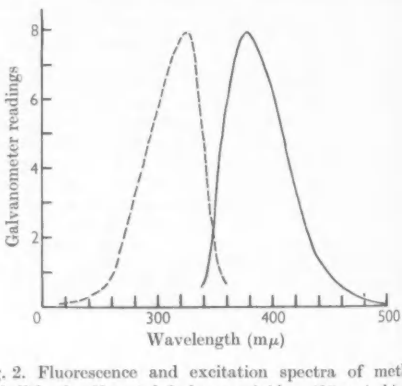

Fig. 2. Fluorescence and excitation spectra of methylumbelliferyl *N*-acetyl-β-glucosaminide (25 μg./ml.) in 0·15 M-glycine buffer, pH 10·3. ——, Fluorescence spectrum, $\lambda_{max.}$ 378 mμ; - - -, excitation spectrum, $\lambda_{max.}$ 325 mμ.

common with other phenols (Robinson, Smith, Spencer & Williams, 1952), the methylumbelliferyl anion shows an absorption peak sufficiently different from that of the glycoside to enable the absorption maximum to be used to determine methylumbelliferone in presence of the glycoside.

Stability of methylumbelliferone and the glycoside. Satisfactory recoveries of methylumbelliferone were demonstrated by Mead *et al.* (1955), when the compound was incubated at 37° and pH 4·6 with various tissue preparations. Levvy *et al.* (1957) found methylumbelliferone to be unstable under a variety of conditions, but particularly when irradiated by sunlight or ultraviolet light. Robinson (1956) found that, even in the cold, methylumbelliferyl β-glucoside decomposed appreciably overnight.

In view of these reports, the stabilities of methylumbelliferone and the *N*-acetyl-β-glucosaminide were investigated by incubation under a variety of conditions followed by fluorimetric assay for methylumbelliferone.

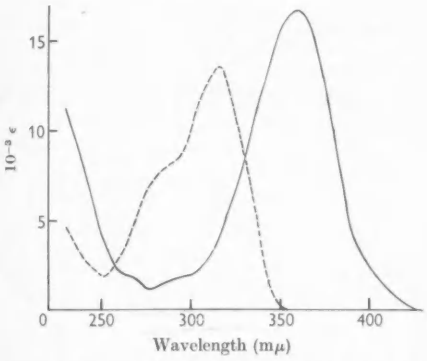

Fig. 3. Absorption spectra of methylumbelliferone and methylumbelliferyl *N*-acetyl-β-glucosaminide in 0·15 M-glycine buffer, pH 10·3. ——, Methylumbelliferone, $\lambda_{max.}$ 360 mμ; - - -, *N*-acetyl-β-glucosaminide, $\lambda_{max.}$ 317 mμ.

Table 1 shows that methylumbelliferone was stable under conditions likely to be encountered during enzyme assay. However, since solutions of methylumbelliferone showed decreased fluorescence on prolonged storage (one aqueous solution showed a 6·5% loss after 21 days at room temperature), fluorescent measurements were carried out against a quinine sulphate standard; the quinine sulphate solution had excellent storage properties.

Solutions of methylumbelliferyl *N*-acetyl-β-glucosaminide slowly liberated methylumbelliferone (confirmed by paper chromatography) on storage at 0–5°; all substrate solutions were therefore prepared freshly before use. The results also showed that small amounts of methylumbelliferone were liberated on incubation under the conditions of enzyme assay; this non-enzymic hydrolysis was linear with time and substrate concentration, but did not vary greatly over the pH range studied (3·8–6·0).

Effect of time of incubation. The effect of time of incubation on the enzymic and non-enzymic hydrolysis of 0·264 mM-methylumbelliferyl *N*-acetyl-β-glucosaminide is shown in Fig. 4. It can be seen that at pH 4·65 and 5·0 the enzymic and non-enzymic liberation of methylumbelliferone was linear with time for at least 90 min. The maximum amount of hydrolysis of the substrate was 0·9%.

Effect of enzyme concentration. The fact that methylumbelliferone could be conveniently estimated (i.e. with the same volumetric technique) in solutions containing 0·0025–11 μg./ml. permitted the enzyme concentration–activity curve to be examined over a wide range of dilution.

Fig. 5 shows that over a 1000-fold range the enzyme concentration–activity curve was linear.

Effect of pH. With methylumbelliferyl *N*-acetyl-β-glucosaminide as substrate, the activity of the testicular enzyme varied with pH as shown in

Table 1. *Stability of methylumbelliferone and methylumbelliferyl N-acetyl-β-glucosaminide*

Solutions of methylumbelliferone (0·22 μg./ml. in water or 0·05 M-citrate buffer, 0·055 μg./ml. in 0·15 M-glycine buffer) and methylumbelliferyl *N*-acetyl-β-glucosaminide (0·264 mM in water or 0·05 M-citrate buffer, 0·066 mM in 0·15 M-glycine buffer) were assayed fluorimetrically (for method see text) before and after incubation under the conditions specified.

Medium	Time (hr.)	Temperature	Loss of fluorescence of methylumbelliferone (%)	Methylumbelliferone liberated from glycoside (μg./ml.)
Citrate, pH 3·83	1·5	37°	<1·4	0·02
Citrate, pH 4·96	1·5	37	<1·4	0·03
Citrate, pH 6·02	1·5	37	<1·4	0·015
Water	18	20–22	<1·4	0·005
Water	18	0–5	<1·4	<0·0025
Glycine, pH 10·3	1·0	20–22	<1·4	<0·0025
Glycine, pH 10·3	18	20–22	2·5	<0·0025

Fig. 6. The pH optimum depended upon substrate concentration, being 5·0 and 4·6 at substrate concentrations of 0·264 and 2·11 mM respectively. Using other substrates for this enzyme, Borooah *et al.* (1961) found pH optima of 4·4–4·8.

Effect of substrate concentration. No direct measurement of the Michaelis constant of an enzyme for a methylumbelliferyl glycoside has been reported. Fig. 7 shows the results of a typical experiment on the effect of various substrate concentrations on the activity of the testicular enzyme. A 256-fold range of substrate concentration (0·0165–4·22 mM) was used, but at no level was the amount of substrate hydrolysed greater than 0·6 %.

It can be seen from Fig. 7 that the curves are different at pH 4·65 and 5·0; both gave straight-line Lineweaver & Burk (1934) plots and the constants derived therefrom were: maximum velocities 1·0 and 0·84 m-moles/30 min., and Michaelis constants 1·22 and 0·89 mM at pH 4·65 and 5·0 respectively. In view of the non-ionic nature of the substrate, these substrate–concentration–pH effects indicate the ionization of an important group at the active site of the enzyme (cf. Dixon & Webb, 1958).

DISCUSSION

Since many glycosidases show little specificity towards the aglycone part of the molecules on which they act, the factors to be considered in the choice of a substrate are its ease of synthesis, stability, solubility and rate of hydrolysis by the enzyme. The stability of the liberated aglycone, and the sensitivity and convenience of the estimation technique are also important. Borooah *et al.* (1961) compared an alkyl and several aryl *N*-acetyl-β-glucosaminides as substrates for testicular *N*-acetyl-β-glucosaminidase; they concluded that the *p*-nitrophenyl glycoside was the most suitable. The high sensitivity of the fluorimetric estimation of methylumbelliferone has been demonstrated by Mead *et al.* (1955); the present work was undertaken to assess methylumbelliferyl *N*-acetyl-β-glucosaminide as a substrate for enzyme assay.

Special routes for the preparation of the β-glucuronide were used by Mead *et al.* (1955), and by Marsh & Levvy (1956): the most general method for the synthesis of methylumbelliferyl 1:2-*trans*-

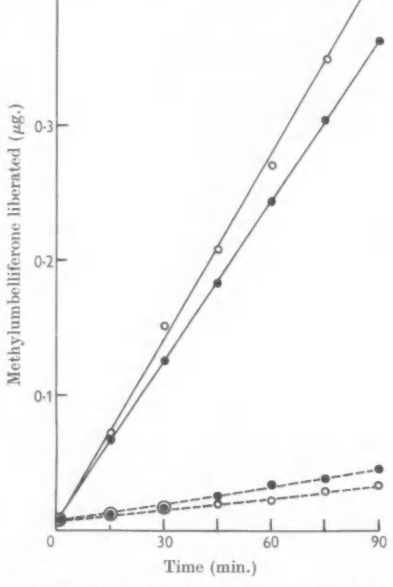

Fig. 4. Effect of time of incubation on degree of hydrolysis. Methylumbelliferyl *N*-acetyl-β-glucosaminide (0·264 mM) was incubated for various periods at 37° in 0·05 M-citrate buffer, pH 4·65 (●) and pH 5·0 (○), in the presence (—) and in the absence (- - -) of *N*-acetyl-β-glucosaminidase.

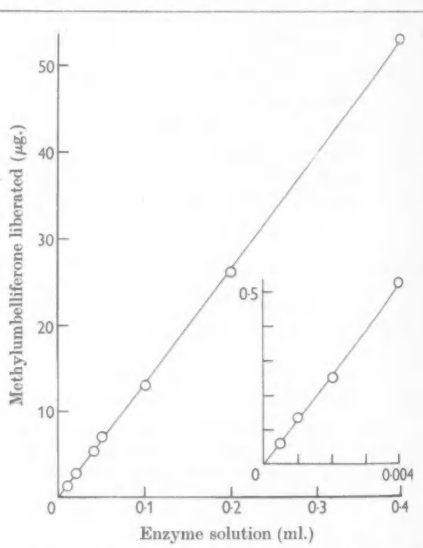

Fig. 5. Effect of enzyme concentration on reaction velocity. Various dilutions of a preparation of *N*-acetyl-β-glucosaminidase were incubated at 37° in 0·05 M-citrate buffer, pH 4·65, for 30 min. Substrate was 2·11 mM-methylumbelliferyl *N*-acetyl-β-glucosaminide; final vol. of reaction mixture was 1·0 ml.; additions of enzyme are expressed as ml. of undiluted preparation. The insert shows a portion of the curve in the region of the origin magnified 40 times.

glycosides seems to be the condensation of an alkaline solution of methylumbelliferone with an acetone solution of the appropriate 1:2-*cis*-acetohalogeno sugar. Under these conditions, yields of the glucoside (Robinson, 1956) and the *N*-acetylglucosaminide were comparable to those of other aryl β-glycosides (cf. Leaback & Walker, 1957).

In preliminary experiments where the activities of enzymes from different sources are to be compared, it is preferable (in the hope of saturating the enzymes present) to use a high substrate concentration. The upper limit of usable substrate concentrations may be determined by the contribution the substrate preparation makes to the blank (cf. Levvy *et al.* 1957). A high fluorescence blank could be due to the fluorescence of the glycoside itself, to the presence of free methylumbelliferone in the preparation or to the non-enzymic release of methylumbelliferone from the glycoside. The information provided by the excitation and fluorescence spectra of methylumbelliferone and the *N*-acetyl-β-glucosaminide permitted the selection of filters which effectively decreased the blank due to scatter and to the fluorescence of the glycoside. Robinson (1956) had difficulty in removing free methylumbelliferone from the glucoside; an efficient method of doing this for the *N*-acetyl-β-glucosaminide has been devised. Under the conditions used in the present work, the non-enzymic liberation of methylumbelliferone from the substrate was not a serious handicap; this effect may, however, limit the use of high substrate concentrations with extremely low enzyme activities.

In kinetic experiments, the use of a wide range of substrate concentration has the advantage of avoiding large graphical extrapolations. Provided that the preparation gives satisfactory blanks, the upper limit of substrate concentration is dependent upon the solubility of the substrate. The glycosides of large aromatic aglycones are usually sparingly soluble, so it is not surprising that the solubility of methylumbelliferyl *N*-acetyl-β-glucosaminide (about 8 mM at 20°) is smaller than that of other aryl *N*-acetyl-β-glucosaminides (cf. Borooah *et al.* 1961). Given the condition that only a small proportion of the substrate should be hydrolysed, the lower limit of substrate concentration is dependent upon the sensitivity of the assay procedure. Owing to the high sensitivity of the fluorimetric method, the methylumbelliferyl glycoside can be used over a wider concentration range than is possible with other, slightly more soluble, aryl glycosides. Thus, in the experiments described in Fig. 7, a 256-fold range of substrate concentration was used: this is in contrast to the 20- to 30-fold range which can be achieved with the phenyl or *p*-nitrophenyl *N*-acetyl-β-glucosaminides (cf. Pugh, Leaback & Walker, 1957; Borooah *et al.* 1961).

The choice of substrate can be influenced by the maximum velocity of enzymic hydrolysis of the substrates concerned; in this respect, there was

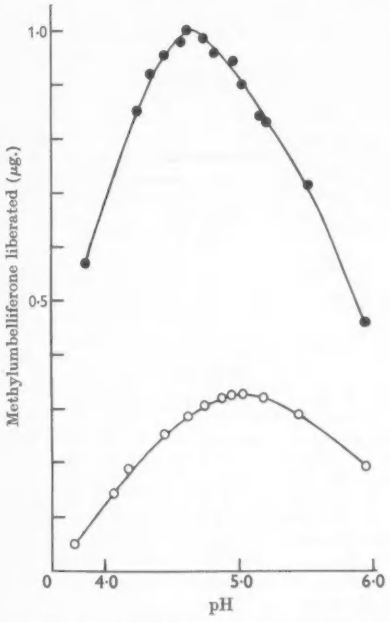

Fig. 6. Effect of pH on reaction velocity. Incubation in 0·05 M-citrate buffer for 30 min. at 37°. Substrate concentration: ○, 0·264 mM; ●, 2·11 mM.

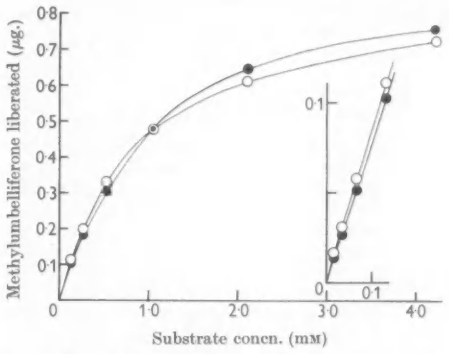

Fig. 7. Effect of substrate concentration on reaction velocity. Various concentrations of methylumbelliferyl *N*-acetyl-β-glucosaminide were incubated with the enzyme for 30 min. at 37° in 0·05 M-citrate buffer, pH 4·65 (●) and pH 5·0 (○). The insert shows portions of the curves in the region of the origin magnified 5 times.

little to choose between the *p*-nitrophenyl and methylumbelliferyl *N*-acetyl-β-glucosaminides, since, with the testicular enzyme, the ratio of the respective maximum velocities was 1:0·95.

The Laurence (1957) fluorimeter proved to be particularly suitable for the present kind of study. In agreement with the findings of Mead *et al.* (1955) and Robinson (1956), the fluorimetric estimation of methylumbelliferone was found to be simple and convenient.

Robinson (1956) pointed out that the fluorimetric method permitted the use of very dilute enzyme preparations and that the high dilution would tend to eliminate the effect of interfering substances. Before assaying very dilute preparations, it may be advisable to establish the form of the enzyme activity–concentration curve; it has been shown here that, with a methylumbelliferyl glycoside, this can be done conveniently with a combination of spectrophotometric and fluorimetric techniques.

The fluorimetric method permits the use of low substrate concentrations with the consequent advantage of conserving substrate; however, the advisability of using low substrate concentrations should be investigated, since results presented here show that characteristic properties of an enzyme (e.g. Michaelis constant, pH optimum) may change under these conditions. Further applications of the fluorogenic method for the estimation of glycosidase activity are being investigated.

SUMMARY

1. 4-Methylumbelliferyl *N*-acetyl-β-glucosaminide has been prepared, and its use as a substrate in the assay of *N*-acetyl-β-glucosaminidase has been described.

2. The range and sensitivity of spectrophotometric and fluorimetric estimations of methylumbelliferone gave the procedure advantages over existing methods.

The authors are greatly indebted to Professor R. T. Williams, Dr D. Robinson and Dr J. N. Smith for advice, and use of the spectrofluorimeter. This work was made possible by Research Grants from the Nuffield Foundation and London University.

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Purification and Specificity of Pancreatic Elastase

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For the determination of the amino acid sequences in proteins, the most useful reagents for the initial breakdown of the molecules are the proteolytic enzymes. Trypsin and chymotrypsin are the most generally used because of their high degree of specificity and because they have different specificities from one another. It would be useful to have other enzymes having complementary specificities which could be used in conjunction with trypsin and chymotrypsin. During work on the structure of insulin (Ryle, Sanger, Smith & Kitai, 1955) it was found that, when insulin was treated with crude pancreatic extracts,

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several bonds that were outside the specificity range of trypsin and chymotrypsin were split. This suggested that one or more other proteolytic enzymes were present and an attempt was made to isolate them and determine their specificities by using the fractions A and B of oxidized insulin as substrates. Evidence was obtained for the presence of an enzyme which splits bonds involving the carboxyl groups of the neutral amino acids with large aliphatic side chains (e.g. leucine and valine). This enzyme appeared to be responsible for many of the splits which were not catalysed by trypsin or chymotrypsin and proved to be identical with elastase. The present paper describes a method for the purification of elastase by using carboxymethylcellulose and describes studies on its specificity with fractions A and B of oxidized insulin.

Elastase was first reported to be present in the pancreas by Balo & Banga (1950). That it was a proteolytic enzyme was indicated by the work of Partridge & Davis (1955) who showed that the dissolution of elastin by elastase resulted in the splitting of peptide bonds. Further evidence was presented by Lewis, Williams & Brink (1956) and Grant & Robbins (1957). The latter workers also showed that although the proteolytic activity of their partially purified elastase was high, its trypsin and chymotrypsin activity as measured against synthetic substrates was low.

MATERIALS

Crystalline α-chymotrypsin and trypsin were obtained from Novo Terapeutisk Laboratorium, Copenhagen, and crystalline carboxypeptidase was obtained from Worthington Biochemical Corp., Freehold, N.J., U.S.A. Carboxymethylcellulose was prepared by the method described by Ellis & Simpson (1956). Pancreatin batch no. CHS/S6 was a gift from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A. Powdered elastin was a gift from Dr S. M. Partridge of Cambridge. This elastin had been prepared by the method of Partridge & Davis (1955) and was essentially free from collagen and carbohydrate.

METHODS

Estimation of elastase activity

Methods for the estimation of elastase activity depend on determining the relative amount of elastin that can be brought into solution. A simplified colorimetric method in which elastin dyed with Congo red is used has been developed. This technique was based on that of Roaf (1908), who used dyed fibrin to measure proteolytic activity.

Powdered elastin (2 g.) was suspended in a saturated aqueous solution (20–30 ml.) of Congo red overnight, and the dyed protein filtered off. The elastin was well washed with water until the washings were clear of dye, and it was then dried by successive washings with acetone and ether.

The colour estimated as $E_{495\ m\mu}$ for completely digested samples was directly proportional to the weight of the sample. The amount of colour corresponding to a given weight of elastin varied slightly from batch to batch. $E_{495\ m\mu}$ for a 0·1% solution of digested dyed elastin was about 1·0.

A sample of the solution to be assayed was added to 3·5 ml. of an elastin suspension in 0·05 M-Na_2CO_3–HCl buffer (pH 8·8) in a 15 ml. centrifuge tube. The weight of elastin in suspension was 4–6 mg./tube. The amount of digestion was estimated by centrifuging down the undissolved elastin and determining $E_{495\ m\mu}$ of the supernatant solution in a spectrophotometer. The undissolved elastin was then resuspended in the supernatant and the digestion allowed to continue. All assays were done at room temperature.

Purification of elastase

Two preparations were used as starting material for purification of the elastase by chromatography on carboxymethylcellulose.

Euglobulin precipitate from pancreatin. The method used was essentially that described by Lewis *et al.* (1956). Pancreatin (23 g.) was extracted by stirring with 250 ml. of ammonium acetate (0·05 M with respect to ammonia) buffer (pH 4·5) for 3 hr. at 5°. The undissolved material was removed in a high-speed refrigerated centrifuge. The sediment was further extracted with 100 ml. of buffer and recentrifuged. The two supernatants were then bulked, and $(NH_4)_2SO_4$ was added to 45% saturation. After 30 min. the precipitate was removed by centrifuging and washed three times with 0·05 M-ammonium acetate buffer containing 45% of $(NH_4)_2SO_4$. The precipitate was then dissolved in 100 ml. of 0·05 M-Na_2CO_3–HCl buffer, pH 8·8, and dialysed against 5 l. of water for 18 hr. at 5° with two changes of water. The resulting euglobulin precipitate was centrifuged down, washed three times with water and freeze-dried.

Crystalline elastase. As the yield of elastase which could be extracted from the pancreatin was low, crystalline elastase was prepared from trypsin (1–300) (Nutritional Biochemicals Corp.) as the starting material, by the method described by Lewis *et al.* (1956). The preparation gave a good yield of needle-shaped crystals, the average yield from 100 g. of trypsin (1–300) being 500 mg. of crystals over five preparations.

Chromatography on carboxymethylcellulose

The material to be fractionated was applied to the column in ammonium acetate buffer of low molarity. Elution was carried out by increasing the NaCl concentration of the developing buffer, and samples (5 ml.) were collected. Protein concentration was estimated from the extinction coefficient at 280 mμ. The molarity of the effluent was determined by reading the resistance of samples (10 ml.) in a conductivity cell (Mullards Ltd., Mullard House, Torrington Place, London, W.C. 1) and relating this to a standard curve. Elastase activity was determined with dyed elastin as described above. The curves shown in the elution diagrams indicate only the presence of elastase. The figures refer to the solution of Congo red-dyed elastin per 0·1 ml. of eluent in 4 ml. of carbonate at an arbitrary time.

The chymotrypsin and trypsin activity was estimated by using the synthetic substrates *N*-acetyl-L-tyrosine ethyl

ester and benzoyl-L-arginine ethyl ester. These estimations were carried out at pH 8 and 25° with an autotitrator and compared with standard amounts of trypsin and chymotrypsin.

Action of elastase on the fractions of oxidized insulin

Digestion of the fractions A and B of oxidized insulin was carried out in an autotitrator (Jacobsen, Leonis, Linderstrøm-Lang & Ottesen, 1957) at pH 8·8 and 25°. The peptides were fractionated by paper ionophoresis (Michl, 1951; Ryle *et al.* 1955) with a potential gradient of 40 v/cm. Buffers at pH 3·5, 6·5 (Naughton, Sanger, Hartley & Shaw, 1960) and 9·1 were used. The latter was prepared by addition of NH_3 to a 2% soln. of $(NH_4)_2CO_3$. The bands were located by dipping narrow guide strips cut from the edge of the paper in a 0·25% soln. of ninhydrin in acetone, and were examined for the presence of arginine, histidine and tyrosine by the colorimetric methods described in Block, Durrum & Zweig (1955). The material from each band was subjected to hydrolysis with 5·7 N-HCl in sealed capillary tubes at 105° for 18 hr., and the amino acids were identified by paper chromatography in butanol–acetic acid–water (4:1:5, v/v) and in phenol–NH_3. *C*-terminal residues of some of the peptides were identified by the use of carboxypeptidase: a carboxypeptidase suspension (2 μl.) and 0·066 M-Na_2HPO_4 (10 μl.) were added to 0·2 ml. of water, and 10 μl. of 0·1 N-NaOH were added to dissolve the carboxypeptidase. This was neutralized by the rapid addition of 10 μl. of 0·1 N-HCl, and the pH of the solution was adjusted to 7·8. 10 μl. of this solution was added to a 0·2 ml. solution of the peptide, and the pH adjusted to 7·8. After incubation (2–6 hr.) of the solution the amino acids released were identified by paper chromatography.

RESULTS

Estimation of elastase activity

In experiments in which the rate of dissolution of elastin was studied with various concentrations of elastase it was found that at low concentrations there was a 'lag phase' during which no elastin was dissolved, and there appeared to be no simple relationship between the amount of elastin dissolved and the concentration of elastase. The procedure cannot be regarded, therefore, as an accurate quantitative method for the estimation of elastase. However, Hall & Czerkawski (1959) and Banga, Balo & Horvath (1959) found similar effects when using different assay procedures.

The colorimetric method was very useful for column chromatography where a large number of samples had to be assayed, as it gave a visual indication of which tubes had elastase activity. The readings of $E_{495\,m\mu}$ gave a rough assay of the amount of elastase.

Experiments were made to ascertain whether agitation of the tube during digestion had any effect on the rate of reaction, but none was observed. This is probably due to the fact that elastase is adsorbed on elastin (Grant & Robbins, 1957), so no shaking is required to produce intimate contact of the enzyme and substrate.

Purification of elastase

Fig. 1 shows the elution curve obtained when the 'euglobulin precipitate' from pancreatin was fractionated on carboxymethylcellulose. A peak containing all the elastase activity was eluted off with 0·2 M-buffer. This peak was assayed for trypsin and chymotrypsin activity against the synthetic substrates benzoyl-L-arginine ethyl ester and *N*-acetyl-L-tyrosine ethyl ester. These assays showed the chymotrypsin activity of the peak to be 1·0% of the protein and the tryptic activity less than 0·5%.

The chromatography of crystalline elastase is shown in Fig. 2. As the protein peak had a shoulder on its leading edge which was not elastolytic, chymotrypsin and trypsin assays were done with fractions from the trailing edge of the peak. The chymotrypsin activity was 1–2% of the

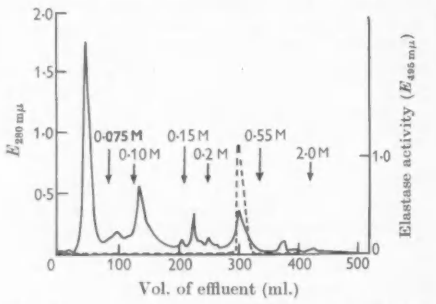

Fig. 1. Chromatography of 'euglobulin precipitate' on carboxymethylcellulose. Euglobulin precipitate (43 mg.) was dissolved in 4·3 ml. of 0·05 M-ammonium acetate buffer (pH 4·5) and applied to the column (10 cm. × 1·5 cm.). The molarity of the buffer was increased in steps by addition as indicated by arrows. —, $E_{280\,m\mu}$; - - -, elastase activity, $E_{495\,m\mu}$.

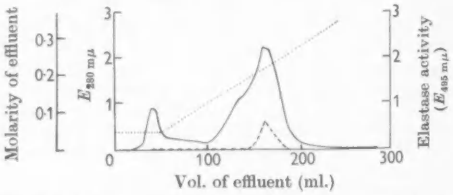

Fig. 2. Chromatography of crystalline elastase on carboxymethylcellulose. Crystalline elastase (100 mg.) was applied in 0·05 M-ammonium acetate buffer (pH 4·6) to the column which was developed with a NaCl gradient. —, $E_{280\,m\mu}$; - - -, elastase activity, $E_{495\,m\mu}$; . . . , molarity of effluent.

protein from the trailing edge whereas the trypsin activity was not detectable, indicating that the trypsin activity of the elastase peak previously prepared from the euglobulin precipitate was probably due to contaminant. 38% of the added protein ($E_{280\ m\mu}$) and approx. 70% of the elastase activity ($E_{495\ m\mu}$) was recovered in the active peak. The material from the trailing edge of this peak appeared to be essentially pure by chromatography and was used in subsequent work. It gave a single peak in the analytical ultracentrifuge.

Reaction of elastase with diisopropyl phosphorofluoridate

The dissolution of elastin in the presence of various concentrations of di*iso*propyl phosphorofluoridate (DFP) is shown in Fig. 3. Complete inhibition was effected by 10^{-4}M-DFP, showing

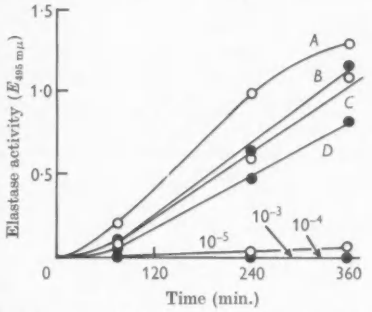

Fig. 3. Inhibition of elastase activity by DFP. Activity was measured by using elastin dyed with Congo red in various concentrations of DFP. Molarities of DFP are: *B*, 10^{-7}; *C*, 10^{-6}; *D*, 10^{-8}. *A*, Control.

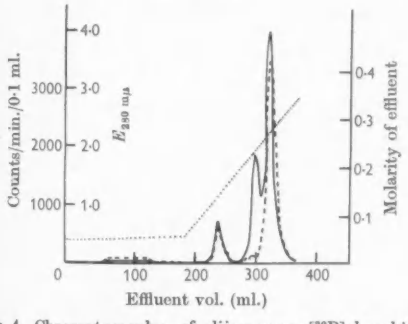

Fig. 4. Chromatography of di*iso*propoxy[^{32}P]phosphinyl derivatives of crystalline elastase on carboxymethylcellulose. Crystalline elastase (100 mg.) was treated with [^{32}P]DFP, dialysed against 0·05M-ammonium acetate buffer (pH 4·5) and applied to the column (40 cm. × 2 cm.). Conditions were as in Fig. 2. —, $E_{280\ m\mu}$; . . ., molarity of effluent; - - -, counts/min./0·1 ml. of effluent.

that elastase, like trypsin and chymotrypsin, is a DFP-sensitive enzyme. Using [^{32}P]DFP, radioactive di*iso*propoxy[^{32}P]phosphinyl-elastase was prepared from crystalline elastase as previously described (Naughton *et al.* 1960). This was subjected to chromatography on carboxymethylcellulose with the result shown in Fig. 4. When the chromatogram is compared with the one from uninhibited elastase shown in Fig. 2, it can be seen that the inactive shoulder in the latter has now been replaced by a separate peak. Two peaks, one showing radioactivity and running in about the same position as untreated elastase and the other non-radioactive, can be seen. The material in the non-radioactive peak was further characterized by the fact that the tubes containing it showed a white precipitate after standing at room temperature or at 0° for about 5 hr. This effect was also noted from the leading edge of uninhibited elastase. The splitting of the shoulder into peaks seems to be due to the fact that elastase has been inhibited by DFP. In four chromatographic separations with uninhibited elastase the inactive peak always appeared as a shoulder, but in every case when di*iso*propoxy[^{32}P]phosphinyl-elastase was used the radioactive elastase and the inactive fraction appeared as distinct peaks. This may be because the di*iso*propoxyphosphinyl group alters the chromatographic rate slightly. No elastase activity was detected after chromatographing di*iso*proxy-[^{32}P]phosphinyl-elastase.

The non-radioactive peak did not react with fraction B of insulin, showing that the contaminant was non-proteolytic. Thomas & Partridge (1960) have found that elastase can be partially activated by cysteine. This is thought to be because the enzyme forms an inactive polymer. Attempts were made to activate the inert peak with cysteine, but as no activity resulted it was concluded that the inactive peak was not caused by an elastase polymer. The small peak which is DFP-sensitive and runs in front of the inactive peak has not been identified.

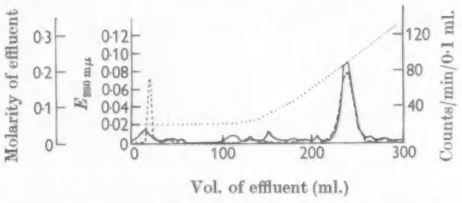

Fig. 5. Chromatography of di*iso*propoxy[^{32}P]phosphinyl derivative of purified elastase on carboxymethylcellulose. Elastase purified by carboxymethylcellulose was allowed to react with [^{32}P]DFP, and a 3 mg. sample was applied to a 2 g. carboxymethylcellulose column as in Fig. 2.

When a sample from the active trailing edge of an elastase chromatogram was inhibited with [^{32}P]DFP and re-run on a 2 g. carboxymethylcellulose column, the elution diagram shown in Fig. 5 was obtained. It can be seen that the protein peak and the radioactive peak parallel one another and there is no trace of an inactive peak running in front. Thus protein from the trailing edge of the elastase peak was not contaminated by the inactive peak.

From the specific radioactivity of the purified di*iso*propoxy[^{32}P]phosphinyl-elastase, it was calculated that the molecular weight of elastase was 28 500, assuming that one mole of DFP reacts with one mole of elastase. The purified di*iso*propoxy-[^{32}P]phosphinyl-elastase was further used to study the amino acid sequence around the reactive serine residue (Naughton *et al.* 1960).

From a nitrogen determination of the purified di*iso*propoxy[^{32}P]phosphinyl-elastase it was found that a 0·1 % solution has $E_{280\,m\mu}$ 1·90.

Action of elastase on fraction B

When the hydrolysis of fraction B by elastase was followed in the auto-titrator there was an initial rapid reaction, which fell off after one equivalent of sodium hydroxide had been added, indicating that one bond was split much more rapidly than the others (Fig. 6). This initial splitting was followed by a much more gradual hydrolysis over a longer period.

Initial rapid reaction. Fraction B (1 μmole) was incubated at 25° with 35 μg. of elastase for 15 min. at pH 8·8. The solution was brought to pH 3 with hydrochloric acid to stop the digestion and dried down in a vacuum desiccator. The hydrolysate was subjected to ionophoresis as a 5 cm. band on a Whatman no. 1 filter paper at pH 3·5 and 40 v/cm. for 1·5 hr. The marker strip showed three bands all moving towards the cathode. Two weak bands moved a distance of 3 and 11 cm. from the origin and the strong band a distance of 6–7 cm. The strong band was a mixture of peptides which could not be separated by ionophoresis at pH 3·5 or 6·5. Ammonium carbonate was found to be a satisfactory volatile buffer which would give a pH high enough to discharge the imidazole ring. The strong band was eluted from the 'pH 3·5' strip and subjected to ionophoresis as a 5 cm. band on Whatman no. 1 paper at pH 9·1, 40 v/cm. for 0·75 hr. Under these conditions two peptide bands were found. The more acidic (BH) gave a positive test for histidine. The slower moving band (BA) gave a positive test for arginine and tyrosine, but a negative one for histidine. The amino acid composition of the bands was determined by paper chromatography. The results, which are shown in Table 1, indicate that peptide BH is derived from the *N*-terminal end of fraction B and BA from the *C*-terminal end. Since peptide BH contains alanine but no tyrosine the splitting must have occurred in the region of the Ala. Leu. Tyr sequence (positions 14–16, Fig. 8).

On treating the peptide BH with carboxypeptidase, leucine and alanine were released. Therefore the rapid split in the B chain of insulin was between leucine and tyrosine residues in positions 15–16. The BA peptide released alanine only under the action of carboxypeptidase, which is consistent with a *C*-terminal sequence of Pro. Lys. Ala.

When a sample of peptide BA was treated with trypsin, a small amount of a peptide having the structure Cy-SO_3H. Gly. Glu. Arg was obtained as well as the anticipated peptide Tyr. Leu. Val.-Cy-SO_3H. Gly. Glu. Arg, indicating that splitting with elastase had also occurred at the Val.-Cy-SO_3H bond and that BA contained two peptides with tyrosine and cysteic acid *N*-terminal residues respectively.

Prolonged digestion of fraction B. Fig. 6 is a record of the alkali uptake when fraction B was digested with elastase. After the initial rapid

Table 1. *Amino acid composition of peptides* BH *and* BA

× ×, × × × and × × × × have the same significance as in Sanger & Tuppy (1951).

Amino acid	Strength in hydrolysate of Peptide BH	Peptide BA
Cysteic acid	× × ×	× ×
Aspartic acid	× × ×	.
Glutamic acid	× × × ×	× × × ×
Histidine	× × × ×	.
Serine	× × ×	× × ×
Alanine	× × ×	× × ×
Tyrosine	.	× ×
Valine	× × ×	× ×
Phenylalanine	× ×	× × ×
Leucine	× × × ×	× × ×
Lysine	.	× × ×
Arginine	.	× × ×
Threonine	.	× ×
Proline	.	× × ×

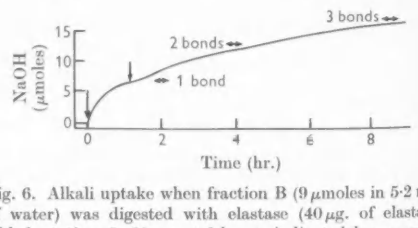

Fig. 6. Alkali uptake when fraction B (9 μmoles in 5·2 ml. of water) was digested with elastase (40 μg. of elastase added at time 0, 80 μg. at 1 hr. as indicated by arrows). Samples were removed at various times; the lines indicating bonds split (↔) have been adjusted to allow for this.

splitting over the first 60 min. there is a gradual slow further splitting. Clearer results were obtained when the isolated BA and BH peptides were hydrolysed separately, and the resulting mixtures fractionated by ionophoresis at pH 3·5. Fig. 7 is an ionophoresis diagram of hydrolysates of fraction B and peptides BA and BH obtained in this way. The structure of the various peptides is shown in Table 2. These were deduced largely from the amino acid composition of hydrolysates of the bands and from the known structure of fraction B. The unchanged BA and BH peptides move at a rate corresponding to band 7. In the ionophoresis diagram of the BH peptide shown, the digestion had not proceeded long enough to produce the peptides due to splitting of the Leu.Cy-SO_3H bond (positions 6–7). The points of cleavage are summarized in Fig. 8.

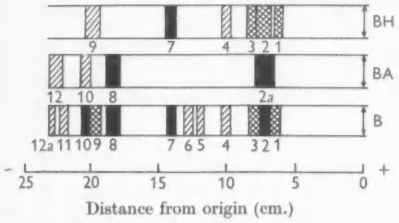

Fig. 7. Ionophoresis diagrams of elastase digests of fraction B and peptides BH and BA. Peptides BH and BA obtained from two μmole samples of fraction B, purified by ionophoresis at pH 3·5 and pH 9·0, and digested with 20 μg. of elastase at pH 8·9 for 24 hr. Ionophoresis pH 3·5, 40 v/cm., 2 hr. (see Table 2).

Action of elastase on fraction A

The reaction of elastase with the A chain of insulin as recorded in the auto-titrator did not show a curve like that for the B chain where one bond was split preferentially. Instead there was a slower reaction which decreased very gradually indicating that a number of bonds were being hydrolysed at equal rates. The peptide pattern obtained from such a hydrolysate gives a complex number of peptides as indicated in Fig. 9. The bands shown in Fig. 9 were eluted and their amino acid composition was determined (Table 3). Band no. 1 was eluted and refractionated by ionophoresis at pH 9, 40 v/cm. for 1 hr. when it gave three bands, 1 A, 1 B, and 1 C. Band A was eluted and refractionated by ionophoresis at pH 6·5, when it gave two bands, A 1 and A 2. Peptides A 2, B and I cannot be

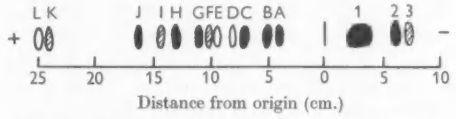

Fig. 9. Ionophoresis diagram of peptides produced by the action of elastase on fraction A. Fraction A (17 mg.) in 2 ml. of 2% $(NH_4)_2CO_3$ buffer (pH 9) treated with 35 μg. of elastase for 18 hr. Ionophoresis on Whatman no. 52 paper, pH 3·5, 35 v/cm., 2 hr. in the apparatus of Gross (1955).

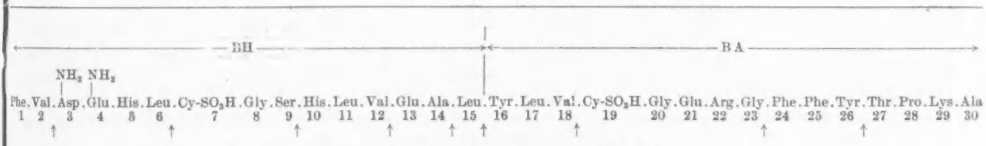
BH
BA
NH2 NH2
Phe.Val.Asp.Glu.His.Leu.Cy-SO3H.Gly.Ser.His.Leu.Val.Glu.Ala.Leu.Tyr.Leu.Val.Cy-SO3H.Gly.Glu.Arg.Gly.Phe.Phe.Tyr.Thr.Pro.Lys.Ala
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30

Fig. 8. Specificity of elastase on fraction B.

Table 2. *Peptides obtained from prolonged digestion of fraction* B *with elastase*

Band (Fig. 7)	Probable structure	Remarks
1	Leucine + valine	
2	Glu.Ala + Glu.Ala.Leu	From BH
2*a*	Cy-SO_3H.Gly.Glu.Arg.Gly	From BA yellow ninhydrin colour
3	Val.Glu.Ala.Leu	
4	Phe.Val.Asp-NH_2.Glu-NH_2.His.Leu.Cy-SO_3H.Gly.Ser	Serine liberated with carboxypeptidase
5	Unidentified	Positive histidine reaction
6	Unidentified	Positive arginine and tyrosine reactions
7	Unchanged BH	
8	Phe.Phe.Tyr.Thr.Pro.Lys.Ala	
9	His.Leu.Val.Glu.Ala	Alanine released with carboxypeptidase
10	Tyr.Thr.Pro.Lys.Ala	
11	Phe.Val.Asp-NH_2.Glu-NH_2.His.Leu	
12	Thr.Pro.Lys.Ala	
12*a*	Thr.Pro.Lys.Ala + Asp-NH_2.Glu-NH_2.His.Leu	Time of hydrolysis of BH too short to identify Asp-NH_2.Glu-NH_2.His.Leu

Table 3. *Peptides obtained by digestion of fraction* A *with elastase*

Band (Fig. 9)	Probable structure
1A	Alanine + Leucine
1B	Valine
1C	Tyrosine
2	Gly.Ileu
3	Gly.Ileu.Val
A1	Tyr.Glu.Leu
A2	Tyr.Glu.Leu.Glu.Asp.Tyr.Cy-SO_3H.Asp
B	Ser.Leu.Tyr.Glu.Leu.Glu.Asp.Tyr.Cy-SO_3H.Asp
C	Ser.Val.Cy-SO_3H.Ser.Leu
D	Glu.Asp.Tyr.Cy-SO_3H.Asp
E	Unidentified
F	Ser.Val.Cy-SO_3H.Ser.Leu.Tyr.Glu.Leu.Asp
G	Ser.Val.Cy-SO_3H.Ser
H	Gly.Ileu.Val.Glu.Glu.Cy-SO_3H.Cy-SO_3H.Ala
I	Gly.Ileu.Val.Glu.Glu.Cy-SO_3H.Cy-SO_3H.Ala.Ser.Val.Cy-SO_3H.Ser
J	Glu.Glu.Cy-SO_3H.Cy-SO_3H.Ala.Ser
K	Glu.Glu.Cy-SO_3H.Cy-SO_3H.Ala.Ser.Val.Cy-SO_3H.Ser
L	Cy-SO_3H.Cy-SO_3H.Ala.Ser.Val.Cy-SO_3H.Ser

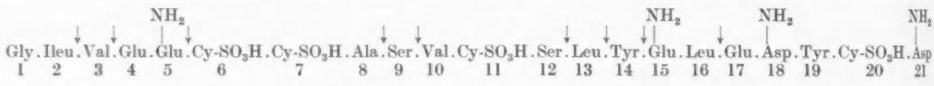
NH2
NH2
NH2
NH2
Gly.Ileu.Val.Glu.Glu.Cy-SO3H.Cy-SO3H.Ala.Ser.Val.Cy-SO3H.Ser.Leu.Tyr.Glu.Leu.Glu.Asp.Tyr.Cy-SO3H.Asp
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21

Fig. 10. Specificity of elastase on fraction A.

regarded as being identified with certainty. From the peptides produced it can be seen that the main splits in fraction A are as shown in Fig. 10.

DISCUSSION

Chromatography on carboxymethylcellulose was a useful method of purification of elastase and gave rise to preparations which are probably purer than any previously reported. Owing to the inaccuracy of the assay method, it is not possible to give precise figures for the degree of purification attained, but the results did indicate that there was no great loss of activity on chromatography and that the material from the active peak was more active than the 'crystalline' elastase. The main evidence for purity must, however, rest on the chromatographic results. Preparations of 'crystalline' elastase were shown to be contaminated with two non-elastolytic components which run in front of the active elastase. The slower of these is not separated clearly from the elastase peak but can be seen as a separate peak when the di*iso*propoxyphosphinyl derivative is chromatographed. The material from the trailing edge of the main elastase peak does not contain this contaminant and was considered to be pure elastase; it was used for the specificity studies. It had no detectable tryptic activity as measured on benzyl-L-arginine ethyl ester and only 1–2% activity on *N*-acetyl-L-tyrosine ethyl ester which is probably an inherent property of the elastase rather than due to chymotryptic activity.

The reaction of the elastase with the A and the B chains of insulin showed that the enzyme can attack a wide variety of peptide bonds involving neutral amino acids having aliphatic side chains. The digestion of elastin by elastase can be explained in terms of this specificity as elastin contains a high proportion of amino acid residues possessing hydrocarbon side chains (Newman, 1949; Partridge & Davis, 1955).

Although elastase does not have a narrow specificity it is a potentially useful enzyme for amino acid sequence studies. It has a very different specificity from trypsin and chymotrypsin and may be useful for splitting peptide chains which are not attacked by these two enzymes as well as for further degradation of larger peptides.

SUMMARY

1. Elastase was purified by chromatography on carboxymethylcellulose.

2. With the fractions A and B of oxidized insulin as substrates it was shown that the purified elastase splits bonds adjacent to the neutral amino acids.

We wish to thank Dr B. C. Saunders for a gift of di-*iso*propyl phosphorofluoridate, Dr D. R. Davis for isotopic DFP and Dr S. M. Partridge for pure elastin.

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Ultracentrifugal Studies of Rat, Rabbit and Guinea-Pig Serum Albumins

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Rat, rabbit and guinea-pig serum albumins have been used in many investigations, but no systematic study of their behaviour in the ultracentrifuge has been reported, except for a short preliminary account of part of the present work (Charlwood, 1959) and a graph showing the dependence on concentration of the sedimentation coefficient of rat albumin at pH 7·5 (Anderson, Canning, Anderson & Shellhamer, 1959). Some measurements have now been completed with the intention of defining the molecular weights of the albumins as accurately as possible, of discovering whether different methods of preparation give essentially the same product and whether the sedimentation coefficients are dependent on pH in the same manner as those of bovine, human and horse albumins (Charlwood & Ens, 1957). In assessing possible differences between preparations, it was necessary to obtain an estimate of the reproducibility of measurements and of the probable magnitudes of errors entering into the various factors concerned. A stock preparation of human albumin, designated A (Charlwood, 1954), was used for many of these experiments.

METHODS

Protein isolation. Some samples of albumin were obtained by electrophoresis of diluted serum in veronal buffer (pH 8·6, *I* 0·1) in the Tiselius apparatus, with automatic control of the compensator, as described by Charlwood (1954) and Warren & Charlwood (1953). Some sera were subjected to preliminary salt fractionation, globulins being mostly removed by saturation with magnesium sulphate followed by filtration (Popják & McCarthy, 1946).

Two organic solvent systems were used for serum fractionation. In the first, 10 vol. of aq. ethanol (96%, v/v, of the alcohol) containing 1% (w/v) of trichloroacetic acid were slowly mixed with 1 vol. of serum at room temperature (Delaville, Delaville & Delaville, 1954). After 30 min., either at room temperature or in the cold (5°), the mixture was centrifuged. The supernatant was dialysed under pressure in the cold against several changes of water. After removal of any precipitate by centrifuging, the albumin solution was dialysed against a suitable buffer. This was veronal (pH 8·6, *I* 0·1) if electrophoresis was to follow, or sodium acetate (pH 4·5, *I* 0·1) if the next stage was to be ultracentrifuging. Solutions not clear at this stage were treated as described below. In some initial experiments the preliminary dialysis against water was omitted, but this proved inadvisable (see Results section). The second method of isolation involving an organic solvent was the same as the previous one, except that the trichloroacetic acid–ethanol system was replaced by 0·4 M-tartaric acid in aq. methanol (90%, v/v, of the alcohol) (Michael, 1958).

When a preparation, made in an organic solvent system, yielded an opalescent aqueous solution which could not be clarified in the ordinary centrifuge, it was often possible to remove the unwanted impurity by centrifuging for 15–30 min. at 39 460 rev./min. in the Spinco preparative rotor SW39. Some electrophoretically separated rat-albumin solutions that contained soluble materials of higher sedimentation coefficients were treated similarly for a period of several hours; this was calculated to bring down the larger molecules but to leave a high proportion of the albumin in the middle of the tubes, whence it could be removed by syringe. This method of fractionation was also applied to one freeze-dried sample of rat albumin that had originally been prepared by the trichloroacetic acid–ethanol method. Many preparations were made on a scale sufficiently large for a comparison to be possible between freeze-dried material and a sample of the same batch which had remained in solution throughout.

Protein quality control. All albumin preparations were examined in the Spinco ultracentrifuge (model E) at high speed (59 780 or 52 640 rev./min.) at a protein concentration of 0·7–1·2%, usually in 0·15M-potassium chloride containing sodium phosphate buffer (pH 6·4, I 0·05). Samples prepared by electrophoresis were checked by examination in the microcell of the Tiselius apparatus at a concentration of 0·8–1·5% in the same buffer as was used for the separation. Many of the other preparations were also analysed in veronal buffer (pH 8·6, I 0·1) in the Tiselius apparatus. When the quantity was more limited, electrophoresis on paper was sometimes preferred. This was done in the manner of Jencks, Jetton & Durrum (1955). The three rat and four rabbit albumins indicated in Table 1 and rat albumin EL/S (see Fig. 1*b*), were examined by immunoelectrophoresis with a combination of the techniques of Grabar & Williams (1953) and Wieme (1959).

Molecular-weight measurements. These were done in the Spinco ultracentrifuge (model E) at low speed (8210 or 12 590 rev./min.), with albumin solutions of concentration 0·6–0·8% previously dialysed against buffer; the buffer was usually sodium phosphate (pH 6·4, I 0·05) containing 0·15M-potassium chloride. Five photographic exposures were recorded at 8 min. intervals, commencing about 30 min. after the ultracentrifuge had attained operating speed. Schlieren bar angles were in the range 45–65°.

The refractive index increment between buffer and protein solution was measured in a differential refractometer of the type described by Cecil & Ogston (1951) at the wavelength used for recording the sedimentation (λ 546 mμ). The refractometer was carefully calibrated with potassium chloride solutions, by using the results of Gosting (1950). From the known constants of the optical system, refractive increments were used to calculate equivalent peak areas. Although confirmatory synthetic-boundary-cell experiments were made occasionally, refractometer measurements were the preferred method for determining the initial concentration.

Enlarged tracings of the curves obtained with the ultracentrifuge were analysed according to the procedures of Charlwood (1957*b*), and molecular weights were calculated by the method of Archibald (1947), from the relation

$$M = \boldsymbol{R}T\,(\partial c/\partial x)_{x=x_0}/c_{x_0}x_0\omega^2(1-\bar{v}\rho), \qquad (1)$$

where c_{x_0} is the protein concentration at the meniscus, x_0 the distance of the meniscus from the axis of rotation, and the other symbols have their usual significance. Partial specific volumes, $\bar{v}$, were taken as 0·736, the value for human albumin near the isoelectric point (Charlwood, 1957*a*). The molecular weight of a sample was taken as the mean value of M derived from the five exposures.

Sedimentation velocity measurements. These were made at 59 780 or 52 640 rev./min., considerable economy of time being effected by the use of wedge window cells. Below pH 5, cells with Kel-F (polychlorotrifluoroethylene) plastic centre-pieces were used for all experiments. The protein concentration was normally about 0·35% and the total ionic strength of solutions was 0·2, of which 0·15 was contributed by potassium chloride. This system was adopted to decrease as much as possible the specific ion effects which have been studied by Kronman & Foster (1957) and Pedersen (1958). Other details were as given by Charlwood & Ens (1957). In addition to the buffers previously used, several sodium citrate buffers were made up covering the range pH 3–4·5. In a few instances the effect of increasing the buffer strength, or using a different protein concentration, was determined.

The ideal of a series of measurements at different protein concentrations at each pH value and extrapolation would have involved a prohibitive amount of work. The measurements made at higher and lower protein concentrations confirmed that concentration dependence of $S_{20,w}$ was in no case so great that conclusions would be materially affected by use of the standard concentration of about 0·35% of protein.

Results were all corrected to water at 20° in the usual way, and expressed in Svedberg units. Correction factors were calculated from viscosity and density information given by Svedberg & Pedersen (1940), Gosting (1950), Lyons & Thomas (1950) and Levien (1955). Independent measurements were made of the viscosity and density (at 25·00±0·02°) of the acetate buffers at pH 3·4 and of the citrate buffers. Viscosity measurements were done in a no. 50 Cannon-Fenske type viscometer (British Standard 188: 1957), and densities in a specially designed flask of about 18 ml. capacity. Except for one acetate buffer (0·15M-potassium chloride + 0·05 ionic strength acetate, pH 3·4), the computed densities and viscosities agreed so closely with the experimental determinations that alterations were unnecessary. As the measured viscosity of this acetate buffer was about 1% higher than the computed value, an appropriate correction was made. In calculating $S_{20,w}$ it was assumed that $\bar{v}$ = 0·736 because, although it varies slightly with pH (Charlwood, 1957*a*) even for a buffer density increment of 0·03, 1% error in $\bar{v}$ affects $S_{20,w}$ by less than 0·3%.

RESULTS

Quality of preparations. Rat albumin separated by electrophoresis of serum, although monodisperse electrophoretically, gave rise in the ultracentrifuge to three components (Fig. 1*a*), the fastest of which could be eliminated by preparative ultracentrifuging. However, the main component was still contaminated by the constituent of intermediate sedimentation velocity (Fig. 1*b*). Prior fractionation with magnesium sulphate eliminated these heavier components (Fig. 1*c*). Rabbit and guinea-pig albumins, separated electrophoretically from serum, appeared to be free from these complications.

In those experiments with the organic solvent systems in which the solution was dialysed in the early stages against acetate buffer rather than water, the albumin produced was often contaminated with material of higher sedimentation coefficient (Fig. 2*a*). On the other hand, this did not apply when the alcoholic solution was first dialysed thoroughly against water (Fig. 2*b*), although the preparations when examined by electrophoresis usually showed a small amount of material with the mobility of α-globulin and sometimes β-globulin as well (Fig. 3*a–d*).

The albumins examined by immunoelectrophoresis all gave a strong arc in the usual position,

but, except with rat albumin EL/S, no other definite components were detected, although there was a slight suggestion of traces of α-globulin in the albumins isolated by means of alcohols. Rat albumin EL/S gave a second, but much weaker, arc beneath the main one.

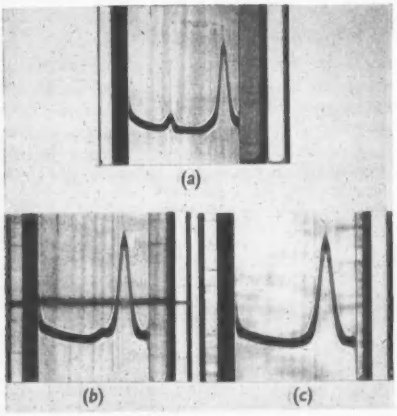

Fig. 1. Ultracentrifuge patterns obtained with rat albumins separated by (*a*) electrophoresis of serum, (*b*) electrophoresis of serum, followed by preparative ultracentrifuging (EL/S) and (*c*) electrophoresis of the fraction of serum not precipitated by saturation with magnesium sulphate. Protein concentration approx. 0·8%. Temperature 20·5°. Sedimentation (right to left) for (*a*) 39 min. at 59 780 rev./min. in veronal buffer, pH 8·6, *I* 0·1, (*b*) 46 min. at 59 780 rev./min. in 0·15 M-KCl + phosphate buffer, pH 6·4, *I* 0·05 and (*c*) 72 min. at 52 640 rev./min. in 0·15 M-KCl + phosphate buffer, pH 6·4, *I* 0·05.

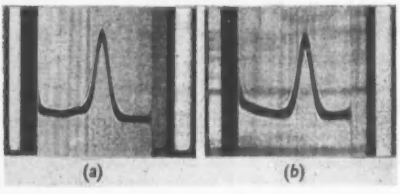

Fig. 2. Ultracentrifuge patterns obtained after precipitation of (*a*) rat serum with ethanol–trichloroacetic acid, followed by dialysis against acetate buffer, pH 4·5, *I* 0·1, (*b*) rabbit serum with methanol–tartaric acid, followed by dialysis against water. Sedimentation (right to left) at 59 780 rev./min. for 82 min. at (*a*) 25·2° in acetate buffer, pH 4·5, *I* 0·1, (*b*) 20·0° in 0·15 M-KCl + phosphate buffer, pH 6·4, *I* 0·05. Protein concentrations (*a*) 1·2%, (*b*) 0·75%.

Molecular weights. The molecular weights of those preparations which appeared by all criteria to be of best quality are given in Table 1. All samples which showed a definite shoulder or faster peak during ultracentrifuging (except at lower pH) have been excluded. The mean values for rat, rabbit and guinea-pig albumin are respectively 64 300, 63 900 and 66 500. The results obtained with human albumin (Table 2) fall within a range of ±2% of the mean (65 200), as expected (see Discussion), whereas in Table 1 the scatter is about ±5%.

The results given in Tables 3 and 4 demonstrate that there is no signifiant difference between molecular weights measured at two ultracentrifuge speeds, nor (with one possible exception) any difference after freeze-drying.

The effect of lower pH on molecular weights is shown in Table 5. The figures, which are weight averages, show that in many cases alterations do occur, but, except for guinea-pig albumin, changes are generally not very large and are reversed when the solution is dialysed back to pH 6·4. Clearly, too, there are often quite marked specific ion effects, citrate especially promoting association.

Sedimentation coefficients. In the present work, values of $S_{20,w}$ for a given solution were usually reproducible within ±1%, often within ±0·5%. The results of measurements on rat and rabbit albumins in glycine, acetate, phosphate and borate buffers are shown in Figs. 4 and 5. Where points coincide, or are too close to be shown distinctly, some have been omitted. With a few possible

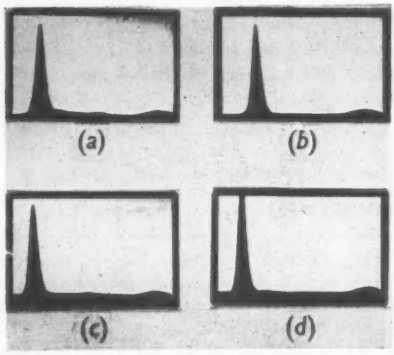

Fig. 3. Ascending electrophoretic patterns at 2·0° in veronal buffer, pH 8·6, *I* 0·1 of (*a*) rat albumin 2 TAR, (*b*) rat albumin 1 TCA, (*c*) rabbit albumin 1 TAR, (*d*) rabbit albumin 1 TCA. Protein concentration approx. 1%. Migration from right to left at 3·1 v/cm. for (*a*) 192 min., (*b*) 168 min., (*c*) 150 min., (*d*) 159 min. For abbreviations see Table 1.

Table 1. *Molecular weights of serum albumins*

Measurements in 0·15 M-KCl + phosphate buffer, pH 6·4, I 0·05.

Sample	Isolation method	Electrophoretic analysis: Paper	Electrophoretic analysis: Tiselius	Molecular weight
Rat				
1 EL	Salt fractionation + electrophoresis	No globulin	No globulin*	62 800†
2 EL	Salt fractionation + electrophoresis	No globulin	No globulin	61 500†
1 TAR	Methanol–tartaric acid	No globulin	—	65 700†
2 TAR	Methanol–tartaric acid	Trace of α-globulin	Some α-globulin, β-globulin*	66 500
1 TCA	Ethanol–trichloroacetic acid	—	No globulin	67 700
2 TCA	Ethanol–trichloroacetic acid	—	Trace of α-globulin*	61 700†
Rabbit				
1 EL	Electrophoresis	—	Trace of α-globulin*	62 800
2 EL	Salt fractionation + electrophoresis	No globulin	No globulin*	60 100†
1 TAR	Methanol–tartaric acid	Some α-globulin, β-globulin	Some α-globulin, β-globulin*	67 900
2 TAR	Methanol–tartaric acid	No globulin	No globulin	63 900
1 TCA	Ethanol–trichloroacetic acid	Trace of α-globulin	Some α-globulin	65 400
2 TCA	Ethanol–trichloroacetic acid	—	Trace of α-globulin, β-globulin*	63 100†
Guinea pig				
1 EL	Electrophoresis	—	No globulin	65 600
2 EL	Electrophoresis	—	No globulin	68 100
1 TAR	Methanol–tartaric acid	—	—	64 900
2 TAR	Methanol–tartaric acid	—	Some α-globulin, β-globulin	67 700
1 TCA	Ethanol–trichloroacetic acid	—	—	69 500
2 TCA	Ethanol–trichloroacetic acid	—	Some α-globulin	64 900†

* Also examined by immunoelectrophoresis (see text).
† Mean of two separate sets of determinations.

Table 2. *Molecular-weight determinations on human albumin*

Buffer	Molecular weight
Acetate, pH 4·6, I 0·2	65 100
	66 000
Acetate, pH 4·5, I 0·05, +0·15 M-KCl	66 400
Phosphate, pH 6·4, I 0·05, +0·15 M-KCl	65 000
	65 800*
	63 800†
	64 800†
	64 800‡

* Mean of two separate sets of determinations.
† After exposure to ethanol–trichloroacetic acid.
‡ After exposure to methanol–tartaric acid.

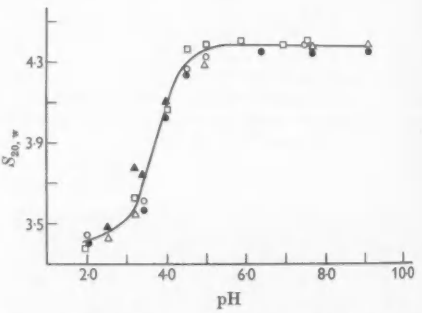

Fig. 4. Sedimentation coefficients of rat albumins as a function of pH. Protein concentration approx. 0·35%. I 0·2, except for rat 3 TCA measurements at I 0·15. ▲, 3 EL; ○, 1 TAR; ●, 1 TAR (freeze-dried); △, 1 TCA (freeze-dried); □, 3 TCA. For abbreviations see Table 1.

exceptions, points lie fairly close to the curves shown, although not within quite such fine limits as if they referred to a single preparation. Any differences between preparations (whether fresh or freeze-dried) are, therefore, negligible so far as these velocity measurements show. The curves are very similar to those obtained under corresponding conditions with bovine, human and horse albumins (Charlwood & Ens, 1957). In the neutral range (pH 6–8) $S_{20,w}$ is close to 4·4.

Below pH 5, doubling the buffer concentration resulted in partial suppression of the decrease of $S_{20,w}$, the general character of the pH-dependence curves remaining unaltered (Figs. 6, 7). Citrate buffers (I 0·2) also decreased the fall in $S_{20,w}$ (Figs. 6, 7). Excellent reproducibility was obtained when duplicate determinations were made immediately after mixing protein and buffer solutions, and after a lapse of some days (Fig. 7).

Guinea-pig albumin preparations, more especially below pH 4, showed measurable differences in behaviour (Fig. 8), but displayed a common characteristic. The general fall in $S_{20,w}$ as the pH decreased was interrupted at about pH 3·6–3·2 by a sharp rise. This rather abrupt transition was accentuated if the protein concentration was decreased or the buffer strength increased (Fig. 9). Although the increase in $S_{20,w}$ coincided with the change from acetate to glycine buffer, similar behaviour was obtained in citrate buffers (Fig. 10) in which the change of environment was much more gradual as the pH altered. In the neutral range the mean value of $S_{20,w}$ is slightly lower (4·3) than for rat and rabbit albumins, but extrapolation of values over a range of concentration gave $S^{\circ}_{20,w}$ 4·46 within experimental error for all albumins.

Table 3. *Comparison of measurements at different ultracentrifuge speeds*

Measurement in 0·15 M-KCl + phosphate buffer, pH 6·4, I 0·05. For abbreviations see Table 1.

	Molecular weight	
Albumin	At 8210 rev./min.	At 12 590 rev./min.
Guinea pig		
2 TCA	65 600	64 200
Human*	67 900	63 600
Rabbit		
1 TAR*	67 400	68 600
1 TCA*	66 300	67 200
2 TCA	62 500	63 700
Rat		
2 TCA	60 000	63 300

* Freeze-dried material.

Table 4. *Molecular weights of albumins before and after freeze-drying*

Measurements in 0·15 M-KCl + phosphate buffer, pH 6·4, I 0·05. For abbreviations see Table 1.

	Molecular weight	
Albumin	Before	After
Guinea pig		
1 EL	65 600	72 800
1 TAR	64 900	68 300
2 TAR	67 700	66 000
1 TCA	69 500	71 100
Rabbit		
1 TAR	67 900	68 000*
1 TCA	65 400	66 800*
Rat		
1 TAR	65 700*	62 500
1 TCA	67 700	67 200*

* Mean of two separate sets of determinations.

DISCUSSION

Nature of contaminants

The attempts to prepare rat albumin by electrophoresis of serum showed that there are other proteins present with a similar mobility but higher sedimentation coefficients; this makes the separation only partly effective. These proteins were assumed to be the two globulins which Grabar & Courcon (1958) detected by immunoelectrophoresis.

Table 5. *Effect of* pH *on molecular weight*

For abbreviations see Table 1.

Albumin	Buffer†	pH	Molecular weight	Molecular weight after redialysis‡
Guinea pig				
2 TAR	Glycine	3·00	95 300	—
2 TAR	Acetate	3·40	75 400	—
2 TCA	Citrate	3·37	117 000	63 100
2 TCA	Citrate	3·92	82 400	—
Human*	Glycine	1·95	67 400	—
	Glycine	2·93	67 800	65 700
	Glycine	2·99	73 100	—
	Acetate	3·34	72 900	—
	Acetate	4·02	64 500	—
Rabbit				
1 TCA*	Glycine	1·92	70 600	71 900
1 TCA*	Glycine	2·92	73 600	71 600
1 TCA*	Acetate	3·37	70 100	73 000
2 TCA	Citrate	3·37	90 300	65 300
Rat				
1 TAR*	Glycine	1·92	57 500	63 200
1 TAR*	Glycine	2·92	63 500	64 000
1 TAR*	Acetate	3·38	61 600	63 900
2 TCA	Citrate	3·39	73 600	65 000

* Freeze-dried material.
† I 0·05, 0·15 M-KCl.
‡ In 0·15 M-KCl + phosphate buffer, pH 6·4, I 0·05.

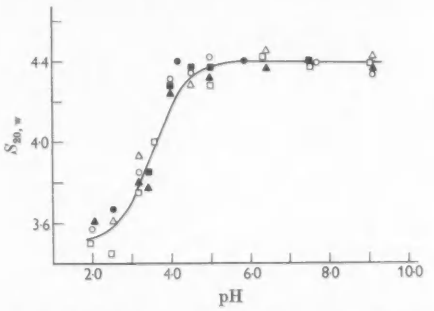

Fig. 5. Sedimentation coefficients of rabbit albumins as a function of pH. Protein concentration approx. 0·35%. I 0·2. ○, 1 EL; ●, 3 EL (freeze-dried); △, 1 TAR (freeze-dried); ■, 3 TAR; ▲, 1 TCA (freeze-dried); □, 3 TCA. For abbreviations see Table 1.

This was confirmed for the component of intermediate sedimentation coefficient by the result of immunoelectrophoresis of rat albumin EL/S. No doubt one also corresponds to the component described recently by Jacox (1959). Although rabbit and guinea-pig albumins isolated by electrophoresis of serum were monodisperse in the ultracentrifuge, they might still have contained material of different molecular weight, but similar sedimentation coefficient. However, with rabbit albumin the failure to detect any other component by immunoelectrophoresis suggested that this possibility could probably be excluded.

The work of Cann, Brown, Singer, Shumaker & Kirkwood (1951) gave an indication of the danger of artifacts arising when serum proteins come in contact with organic solvents, but Schwert (1957) and Ram & Maurer (1958) showed that bovine serum albumin appeared unchanged in its sedimentation velocity, electrophoretic, immunochemical and other properties after exposure to trichloroacetic acid–ethanol mixtures. Table 2 shows that the molecular weight of human albumin is unaltered after similar treatment. Differences in the metabolic behaviour of human albumin isolated with trichloroacetic acid–ethanol (Freeman, 1959) are most probably due to changes brought about during an extra stage of ether precipitation. Immunologically the rat and rabbit albumins tested appeared quite normal. It would thus seem most

Fig. 6. Sedimentation coefficients of rat albumins as a function of pH. Protein concentration approx. 0·35%. □, 1 TAR (freeze-dried) in buffers, I 0·4. Other points refer to citrate buffers, I 0·2, ●, 1 TAR (freeze-dried); ○, 2 TCA (freeze-dried). Dotted line corresponds with curve of Fig. 4. For abbreviations see Table 1.

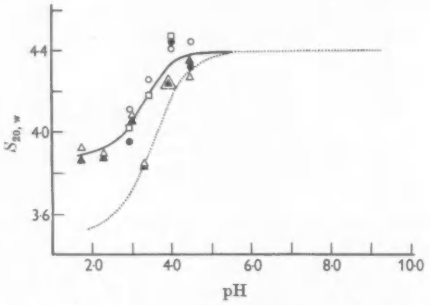

Fig. 7. Sedimentation coefficients of rabbit albumins as a function of pH. Protein concentration approx. 0·35%. □ and ▲ represent respectively 1 TCA (freeze-dried) in buffers, I 0·4, immediately after mixing and about 14 days later. Other points refer to citrate buffers, I 0·2. ○, 1 TAR (freeze-dried); □, 1 TCA (freeze-dried); ● 2 TCA. Dotted line corresponds with curve of Fig. 5. For abbreviations see Table 1.

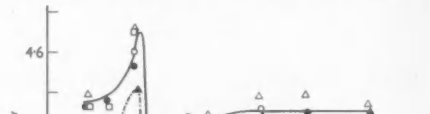

Fig. 8. Sedimentation coefficients of guinea-pig albumins as a function of pH. Protein concentration approx. 0·35%. I 0·2. ○, 2 EL; ●, 1 TAR; □, 2 TAR (freeze-dried); △, 1 TCA (freeze-dried); ▲, 3 TCA. For abbreviations see Table 1.

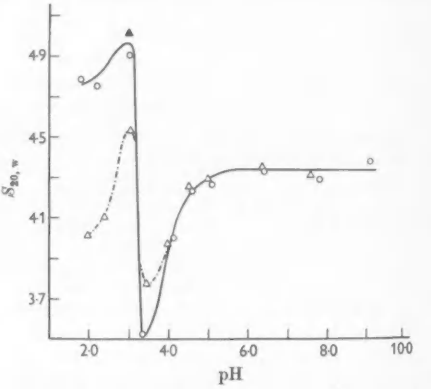

Fig. 9. Sedimentation coefficients of guinea-pig albumins as a function of pH. Protein concentration approx. 0·18%. ▲, 1 TAR (freeze-dried) in buffer, I 0·4; ○, 1 TCA (freeze-dried) in buffers, I 0·4; △, 1 TCA (freeze-dried) in buffers, I 0·2. For abbreviations see Table 1.

likely that protein impurities in the albumins which have been in contact with an alcohol (Table 1) consist, not of altered albumin, but of those globulin fractions which are also soluble. It is known that protein solubility in acid–ethanol mixtures is not a property exclusive to albumins, since, for instance, Chibnall, Mangan & Rees (1958) have shown that insulin and β-lactoglobulin are soluble in ethanol containing hydrogen chloride. The contaminants of the animal albumins behaved on electrophoresis as α- or β-globulins, their proportions varying with the individual sera fractionated.

Accuracy of results

The main assumptions underlying the derivation of equation (1) are that the solution contains only two components, and that it is sufficiently dilute for the ratio of sedimentation and diffusion coefficients of the protein to be nearly the same as at infinite dilution. However, the equation applies under less stringent conditions to a good degree of approximation. In buffer systems, strictly speaking, various other interaction factors ought to be taken into account (Williams, van Holde, Baldwin & Fujita, 1958). Assuming that these are small enough to be neglected under the present conditions, the accuracy of determination of M can be assessed from a consideration of the factors of equation (1).

The factors which are most susceptible to error are c_{x_0}, $(\partial c/\partial x)_{x=x_0}$ and $\bar{v}$. It is unnecessary to know the specific refractive increment of the protein, as this enters into both c_{x_0} and $(\partial c/\partial x)_{x=x_0}$. The bar angle of the schlieren system must be carefully adjusted, as otherwise it leads to errors in c_{x_0} and $(\partial c/\partial x)_{x=x_0}$, which are additive. The value of c_{x_0} depends on the original concentration, c_0, measured in the differential refractometer (estimated error not more than 0·5 %) and on the area below the schlieren curve between the meniscus and the plateau region. This area is measured with an accuracy of about 2 %, but the term proportional to this area is generally only about 30 % of c_0. Thus c_{x_0} is known to an accuracy of about 1·5 % (see Charlwood, 1957*b*). The factor $(\partial c/\partial x)_{x=x_0}$ is proportional to the height above the base-line at which the curve intercepts the meniscus. By using slightly higher speeds, Ehrenberg (1957) was able to reduce the error involved in measuring this intercept, but only at the expense of increased uncertainty in c_{x_0} which is then the relatively small difference of two large numbers. Owing to diffraction effects it is necessary to extrapolate the schlieren curve in the immediate neighbourhood of the meniscus (Ginsburg, Appel & Schachman, 1956). This is mainly responsible for the fluctuations in M (sometimes as great as ±5 %) within a series of five photographs. The error in the mean from this cause should not, however, be more than ±3·5 %. Taking into account errors in c_{x_0}, therefore, differences of M much greater than 5 % between batches ought to be regarded as significant.

The value of $\bar{v}$ used does not affect the relative values of M for different preparations of albumin, although a small error in the assumed value of $\bar{v}$ is approximately trebled in the calculation of the absolute value of M. As $\bar{v}$ is determined mainly by the amino acid composition of a protein, and is nearly the same for bovine, human and horse serum albumins (Charlwood, 1957*a*), errors arising on this account are probably not very great.

It has been assumed above that the protein is monodisperse in the ultracentrifuge. Polydisperse aggregates, or contaminants of moderate size sometimes not easy to detect in velocity ultracentrifuging, will give rise to a spurious molecular weight. As the method yields a weight average, aggregates have a noticeable effect, giving initially too high a value of M, which decreases with time as the solution near the meniscus becomes differentially depleted of larger components (Smith, Wood & Charlwood, 1956). It is theoretically possible for very large aggregates to sediment so fast during the preliminary stages that assumptions about the concentration in the plateau region are no longer correct, and an erroneously low value of M is obtained. However, it is improbable that this would occur undetected. As a check on this

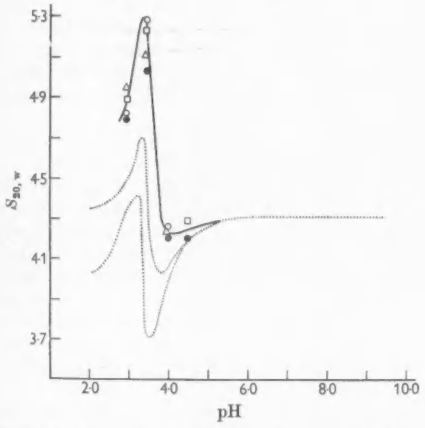

Fig. 10. Sedimentation coefficients of guinea-pig albumins as a function of pH in citrate buffers, I 0·2. Protein concentration approx. 0·18 % for 1 TCA, 0·35 % for the rest. ○, 2 TAR (freeze-dried); △, 1 TCA (freeze-dried); □, 2 TCA; ●, 3 TCA. Dotted lines correspond with curves of Fig. 8. For abbreviations see Table 1.

a comparison was made of the starting material and the centre layer from the rat-albumin solution centrifuged in the preparative rotor SW 39. The molecular weights were indistinguishable. Although, in experiments quoted in this paper, there was never a significant drift of M with time, the fluctuation of $\pm 5\%$ within a series of five exposures could obviously have obscured any small trend.

The agreement between results at different speeds indicates that there is insufficient vibration to cause significant anomalies.

Ultracentrifugal findings

Independent measurements of $S_{20,w}$ and diffusion coefficients are available for the human albumin (Charlwood, 1954). Strict comparison with the present work requires a correction of 2·5 % to the sedimentation coefficients (Charlwood, 1954), $\bar{v}$ to be taken as 0·736, and values of $S_{20,w}$ and $D_{20,w}$ to refer to an albumin concentration of about 0·6 %, corresponding to the mean value of c_{x_0}. The good agreement between M 64 300 thus obtained, and M 65 200 from Table 2 substantiates the reliability of the figures.

The molecular weights of different preparations of the animal albumins varied more than was to be expected on the basis of errors of measurement alone. It must be concluded that this was due to the small amounts of contaminants demonstrated in some cases, and possibly present but not detected in others. The variations seemed sufficiently small for the best estimates of M to be taken as the mean values in Table 1. These differ slightly from the figures of Charlwood (1959), but are probably more nearly correct, being based on a larger number of preparations and more rigorous quality control. The apparently significant small difference between the molecular weight of guinea-pig and the other albumins no longer exists.

The reasonable consistency of M among the preparations obtained by different methods implies that no changes in the state of aggregation of the albumin are involved, in contrast with what probably happens when γ-globulin is exposed to some systems containing ethanol (Cann *et al.* 1951).

There have been reports of aggregation taking place when albumin is stored in the freeze-dried state (Halwer, Nutting & Brice, 1951; Christiansen, Jensen & Marcker, 1957). No aggregates were observed in high-velocity ultracentrifuging of freeze-dried samples when dialysed in phosphate buffer (I 0·05, pH 6·4) containing 0·15 M-potassium chloride, and there was little, if any, evidence of molecular-weight changes. Bovine albumin, dissolved in acetate buffer (I 0·2, pH 4·6) and examined immediately in the ultracentrifuge, although it revealed the usual small proportion of a faster component, showed no sign of the aggregation which would have been expected from the measurements of Christiansen *et al.* (1957). This work, therefore, gives no indication that freeze-drying is responsible for any aggregation of albumins. Nevertheless, traces of contaminants may aggregate in these circumstances and might thus give rise to misleading conclusions.

The general decrease of sedimentation coefficients below about pH 5 is presumably due to the same factors as for bovine and human albumin, i.e. to a general expansion of the molecule due in part to electrostatic repulsion. This is borne out by the observation that increase of ionic strength partly suppresses the fall of $S_{20,w}$, much in the same way as with bovine serum albumin (Reichmann & Charlwood, 1954). The smaller decrease in $S_{20,w}$ in citrate buffers is most likely due to a decrease in the net charge brought about by binding of citrate ions to the protein, since Kronman & Foster (1957) showed that $S_{20,w}$ for bovine albumin decreased as the net charge on the molecule increased. The reproducibility of measurements after a time interval (Fig. 7) shows that, in this instance at least, the changes in protein configuration at lower pH are complete within an hour or two at most.

It was thought that differences between preparations might be revealed by measurements of sedimentation coefficient at lower pH if the ion-binding characteristics differed, a more sensitive test than comparison of molecular weight or sedimentation near neutrality. There were no clear indications of such differences among the rat and rabbit albumins, but guinea-pig albumins were by no means identical in sedimentation coefficients at low pH. However, these differences may be partly a reflexion of the critical dependence of the state of molecular association on pH for guinea-pig albumin.

The interruption in the fall of $S_{20,w}$ below pH 5, which takes place at pH 3·6–3·2, seemed much more likely to be due to the formation of a dimer (or higher aggregate) than to a sudden reversion of the guinea-pig albumin molecule to a more compact shape. This supposition was confirmed by molecular-weight measurements (Table 5), which also indicated that association had already begun above pH 3·6. The failure to observe more than one peak at 0·35 % protein concentration may be due to various causes. Thus, if equilibrium between monomer and dimer is rapid, resolution cannot occur (Gilbert, 1959). Moreover, the lower the protein concentration, the more difficult it becomes to distinguish a given proportion of a second component, although careful re-examination of some of the records did reveal that there was sometimes asymmetry of peaks. If monomer and dimer exist together in comparable amounts only over a very

narrow range of pH, this may not include the limited number of buffers used. The behaviour of guinea-pig albumin is similar to that of bovine albumin, which tends to associate near pH 3 (Charlwood & Ens, 1957), but the effect is more marked here. Human albumin displays a similar tendency (Table 5), but rat albumin did not associate except in citrate buffer (Table 5). The low value of M for rat albumin at pH 1·92 is most probably due to the influence, under these conditions, of the factors which have been generally assumed to be negligible (see comment on Williams *et al.* 1958 above).

The apparent slight increase in $S_{20,w}$ between pH 3·4 and pH 3·2 for rat and rabbit albumins may be a reflexion of a small amount of association, but the change coincides with an alteration from acetate to glycine buffer, the acetate buffer necessitating very large viscosity and density corrections which may not be perfectly valid, especially at I 0·4. However, there does seem to be some suggestion of a similar effect in citrate buffer where the corrections are considerably smaller.

The association of guinea-pig albumin occurs mainly over a narrow range of pH (which is affected slightly by the buffer used), and goes nearer to completion than does the corresponding reaction for other albumins under comparable conditions. If a necessary prerequisite for association is the exposure of a particular site on the molecule, it might be supposed that this would occur at a particular degree of expansion (or extension), and that $S_{20,w}$ would therefore drop to a definite value at this point. However, comparison of results in citrate and other buffers (Fig. 10) is not consistent with this simple interpretation. Further work will therefore be required to elucidate the nature of the mechanism by which the association occurs.

SUMMARY

1. Albumins have been prepared from rat, rabbit and guinea-pig sera by trichloroacetic acid–ethanol and tartaric acid–methanol systems as well as by free electrophoresis. Salt fractionation with magnesium sulphate was sometimes used in conjunction with these methods.

2. The molecular weights of all these albumins, measured near neutrality in the ultracentrifuge, were close to 65 000. Differences were due to a combination of random error and the residual small amounts of globulins. There was no demonstrable effect of freeze-drying on the molecular weights.

3. The pH-dependence of $S_{20,w}$ for rat and rabbit albumins was closely similar to that for bovine, human and horse albumins, $S_{20,w}$ falling steadily from about pH 5 to pH 2. The effect of variations in the environment indicated that electrostatic factors were implicated.

4. The pH-dependence of $S_{20,w}$ for guinea-pig albumin involved a steady fall below pH 5, interrupted by a large increase between approximately pH 3·6 and 3·2. This was shown to be due to dimer formation, but there was not sufficient information to reveal the details of this process.

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A Colorimetric Method for the Estimation of Monoamine Oxidase

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The reaction catalysed by monoamine oxidase is generally represented (Zeller, 1951; Blaschko, 1952) by the equation

$$RCH_2{\cdot}CH_2{\cdot}NH_2 + H_2O + O_2 \rightarrow RCH_2{\cdot}CHO + NH_3 + H_2O_2. \quad (1)$$

Quantitative assay methods have been described based on measurement of the disappearance of substrate, the uptake of oxygen, or the liberation of ammonia, but none of these is entirely satisfactory for studies of the kinetics of the reaction. The most widely used method, measurement of the oxygen consumption, is relatively insensitive and is complicated by secondary reactions which occur, owing to the presence of other oxidizing enzymes, in normal tissue or mitochondrial-monoamine-oxidase preparations. The hydrogen peroxide is decomposed by catalase, which is found in all the tissue preparations, thus reducing the oxygen uptake from 2 atoms/mole of substrate as indicated in equation (1) to 1 atom/mole. The aldehyde may be further oxidized to the corresponding carboxylic acid or to resinous polymeric products; this results in a time-dependent and uncontrolled absorption of further oxygen (Cotzias & Greenough, 1959). This decomposition of the aldehyde may be prevented if aldehyde trapping agents, such as cyanide or semicarbazide, are added to the assay mixture, and under these conditions the oxygen absorption can be limited to 1 atom/mole of substrate (Creasey, 1956) but only at the expense of a further loss in sensitivity. Further, the presence of cyanide ions in the assay mixture may produce a dramatic change in the susceptibility of the enzyme to certain inhibitors (Davison, 1957). Measurement of the ammonia liberated provides a reliable and sensitive assay method but the procedure is rather laborious (Cotzias & Dole, 1951). Measurement of substrate disappearance means there must be an appreciable change in substrate concentration during the assay; this is undesirable for studies of kinetics.

The aldehyde semicarbazone, which is formed quantitatively when the enzymic oxidation is carried out in the presence of an excess of semicarbazide, can be readily converted into the corresponding 2:4-dinitrophenylhydrazone (Richter, 1937). Dinitrophenylhydrazones have an intense colour in alkaline solution (Friedemann & Haugen, 1943), and this offers a convenient means of measuring the enzymic activity. The present paper describes an assay procedure based on this dinitrophenylhydrazone formation. The method is sensitive and can be used over a wide range of substrate concentrations without significant depletion of the substrate during the reaction.

EXPERIMENTAL AND RESULTS

Materials. Guinea-pig-liver mitochondria, prepared by differential centrifuging of guinea-pig liver homogenized in 0·25 M-sucrose (Hawkins, 1952), were used as the enzyme source. The sedimented mitochondria were suspended in 0·1 M-phosphate buffer, pH 7·4 (83 ml. of 0·2 M-Na_2HPO_4 + 17 ml. of 0·2 M-NaH_2PO_4 + 100 ml. of water); 1 ml. of suspension was equivalent to 1 g. wet wt. of liver. This preparation was stored at $-10°$, and showed little loss of activity even after storage for several months.

Tyramine hydrochloride was obtained from L. Light and Co. Ltd., Colnbrook, Bucks.

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p-Hydroxyphenylacetaldehyde 2:4-dinitrophenylhydrazone, required as a colorimetric standard, was prepared as described by Richter (1937) by addition of 2:4-dinitrophenylhydrazine to the product obtained from the enzymic oxidation of tyramine in the presence of semicarbazide. On crystallization from ethanol the compound had m.p. 180° (decomp.) (Richter gives m.p. 183–186° from benzene); light-absorption max. (in ethanol) 358 mμ (ε 23 000). When dissolved in alkali this dinitrophenylhydrazone is at first deep red, but the colour gradually changes to orange–yellow when the solution is kept at room temperature for about an hour. This colour change is accelerated by heat and is complete if the solution is kept at 80° for 10 min. In 0·1 N-NaOH the orange–yellow form is stable at room temperature for several hours, and has a light-absorption maximum at 425 mμ (ε 9500).

Spectra. The u.v. spectra of the dinitrophenylhydrazone in alcohol and alkali and all other extinction measurements were determined with a Unicam SP. 500 spectrophotometer.

Assay procedure. 0·05 M-Semicarbazide hydrochloride, neutralized to pH 7·4 (1·0 ml.), 0·2 M-phosphate buffer (1·6 ml.) and enzyme (1·0 ml. of an approximately tenfold dilution of the stock suspension with buffer) were placed in a loosely stoppered 25 ml. conical flask which was shaken in a water bath at 25°. After 10 min., to allow the flask contents to reach a uniform temperature, 0·1 M-tyramine hydrochloride, neutralized to pH 7·4 (0·4 ml.), was added and the mixture was shaken in the bath for a further 30 min. 0·5 N-Acetic acid (1·0 ml.) was then added to stop the reaction. The flask contents were transferred to a centrifuge tube and heated in boiling water for 3 min. to precipitate the protein which was then removed by centrifuging. 2:4-Dinitrophenylhydrazine in 2 N-HCl (2·0 ml., 0·5 mg./ml.) was added to 2·0 ml. of the supernatant, and the mixture was kept at room temperature for 10 min. The dinitrophenylhydrazone which was formed was extracted with benzene (5·0 ml.), and 4·0 ml. of the benzene layer was then shaken with 0·1 N-NaOH (4·0 ml.). In both extraction steps the mixtures were centrifuged to ensure complete separation of the two layers. The benzene layer was then discarded, and the alkaline layer was heated at 80° for 10 min. to convert the initial red form of the dinitrophenylhydrazone into the orange–yellow form. When cool the solution was transferred to a glass 1 cm. spectrophotometer cell, and its extinction was measured at 450 mμ. After correction for the blank (a parallel run containing water instead of substrate), the extinction gave a direct measure of the enzymic activity.

Batches of six to eight estimations can be conveniently carried out at one time.

The typical results in Fig. 1 show that the extinction increases linearly both with time of incubation and with enzyme concentration. This linear relationship holds up to an extinction of at least 1, and over a time of at least 1 hr.

The reproducibility of the above procedure is illustrated by the following extinction readings (after correction for a blank of 0·084) obtained in five replicate runs: 0·636, 0·618, 0·616, 0·616, 0·606.

Extraction procedures. The double-extraction technique (cf. Friedemann & Haugen, 1943) was adopted, to eliminate as much unchanged 2:4-dinitrophenylhydrazine as possible from the final alkaline solution and so minimize the blank due to the strong colour of alkaline solutions of dinitrophenylhydrazine itself.

The completeness of the extraction of *p*-hydroxyphenylacetaldehyde 2:4-dinitrophenylhydrazone from benzene by alkali is dependent on the pH. Benzene solutions of the crystalline compound were shaken with buffers or aqueous sodium hydroxide over the pH range 11–14, and the concentration of the dinitrophenylhydrazone in both the benzene and alkaline layers was determined by measurement of the extinctions at 360 and 425 mμ respectively. A similar experiment was carried out on 2:4-dinitrophenylhydrazine. With N-sodium hydroxide, almost 100% extraction of the dinitrophenylhydrazone was achieved, but the stronger alkali also produced a marked rise in the blank reading.

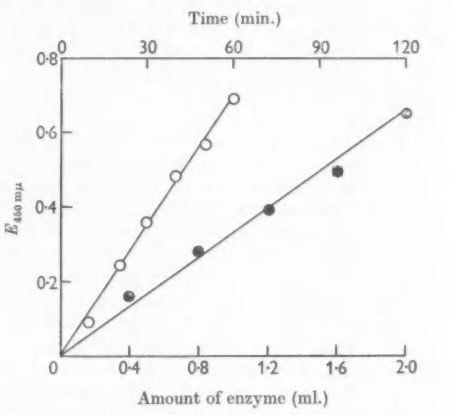

Fig. 1. Dependence of extinction on time and enzyme concentration. ○: Enzyme suspension (0·6 ml. of an eightfold dilution of the stock suspension), tyramine (0·01 M) and semicarbazide (0·0125 M) in a total volume of 4 ml., incubated for various times at pH 7·4 and 25°; ●: enzyme suspension (a 20-fold dilution of the stock suspension), tyramine (0·01 M) and semicarbazide (0·0125 M) in a total volume of 4 ml., incubated for 30 min. at 25° and pH 7·4.

0·1 N-Sodium hydroxide which extracted 91–93 % of the dinitrophenylhydrazone from the benzene gave the most satisfactory compromise between completeness of extraction and minimization of the blank.

The efficiency of the extraction of the dinitrophenylhydrazone into benzene during the assay was investigated as follows. Two flasks were run in parallel as in the assay method, one a test sample with substrate, the other a blank without substrate. After deproteinization, samples from each of the two flasks were mixed in known proportions. 2:4-Dinitrophenylhydrazine was then added and the dinitrophenylhydrazone was extracted as usual, first into benzene and then into alkali. A second extraction of the mixtures with benzene and alkali produced very little further colour, and the ratios of the extinctions of the alkaline extracts from the first and second benzene extractions, after correction for the blanks, showed that the first extraction was about 90 % complete. The corrected extinctions of the first alkaline extracts were directly proportional to the fraction of test sample, and hence to the concentration of dinitrophenylhydrazone in each mixture.

These results, taken in conjunction with the reproducibility of the assay procedure itself and the linear dependence of extinction on enzyme concentration (Fig. 1), show that although the overall extraction process is only about 80 instead of 100 % complete, the extinctions of the final alkaline solutions give a direct measure of the dinitrophenylhydrazone which has been formed.

Stoicheiometry. In one experiment the assay was carried out in Warburg flasks fitted with manometers instead of in the usual conical flasks. The reaction was stopped after 45 min. when 7·2 μg.-atoms of oxygen had been absorbed. The remainder of the assay procedure was then carried out in the normal way. The amount of dinitrophenylhydrazone recovered was 5·8 μmoles. After correction for the incompleteness of the extraction, this represents a total dinitrophenylhydrazone production of about 7·3 μmoles, i.e. 1 μmole of dinitrophenylhydrazone for each μg. atom of oxygen absorbed.

Other factors influencing the colour. Variation in the concentration of dinitrophenylhydrazine used in the assay procedure showed that an increase above the chosen value of 0·5 mg./ml. did not enhance the intensity of the colour formation but merely increased the blank. Any appreciable decrease below 0·5 mg./ml. did, however, cause some diminution in the extinction of the final solution.

The concentration of semicarbazide that was chosen (0·0125 M) was the minimum consistent with efficient trapping of the aldehyde. A large increase in semicarbazide concentration (> 0·05 M) caused a slight decrease in the extinction.

The activity of the enzyme was not significantly affected by variation in the concentration of the tyramine substrate provided this was not decreased below 1 mM. When the reciprocal of the extinction was plotted against the reciprocal of the substrate concentration over the range 1–0·1 mM, a straight line was obtained consistent with the enzymic oxidation obeying the Michaelis-Menten rate equation ($v = k[\mathrm{E}]\,[S]/(K_m+[S])$) with a value of 3×10^{-4} moles/l. for K_m.

DISCUSSION

Some of the difficulties which arise when current methods for monoamine-oxidase estimation are used in studies of kinetics were described in the introduction. Measurement of the rate of aldehyde formation, by first trapping the aldehyde with semicarbazide and then converting the semicarbazone into the corresponding 2:4-dinitrophenylhydrazone, enables many of these difficulties to be avoided. The technique is reasonably quick and straightforward. The method is reproducible and gives satisfactory linear relationships between extinction and enzyme concentration or time, as illustrated in Fig. 1.

The double-extraction procedure is desirable to minimize the amount of unchanged dinitrophenylhydrazine present in the final alkaline solution, the extinction of which is measured on the spectrophotometer. The extraction process does not entirely eliminate dinitrophenylhydrazine, but the small amount left can be adequately corrected for by reference to the blank run. Since the absorption peak of the dinitrophenylhydrazine itself in alkali is at about 390 mμ it was found to be preferable for assay purposes to measure the extinction of the dinitrophenylhydrazone at 450 mμ instead of at the peak wavelength of 425 mμ. This results in only a small decrease in the extinction of the dinitrophenylhydrazone, but to a considerable fall in the blank reading.

When allowance is made for the incompleteness of the two extraction steps, there is a one to one correspondence between the number of μg.atoms of oxygen absorbed and the number of μmoles of dinitrophenylhydrazone formed. If the assay procedure is carried out as described, a final extinction of 0·1 (after correction for a blank of the same magnitude) represents an oxygen uptake of about 2 μl. and the destruction of less than $\frac{1}{200}$ of the tyramine substrate.

SUMMARY

1. A method is described for the estimation of monoamine oxidase based on measurement of the aldehyde formed during the enzymic oxidation of tyramine. The unstable aldehyde is prevented from decomposing by the presence of semicarbazide in

the assay mixture. The semicarbazone so formed is then converted into the corresponding 2:4-dinitrophenylhydrazone. The colour of this material in alkaline solution provides a sensitive measure of the enzymic activity.

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The Biochemistry of Aromatic Amines

8. SYNTHESIS AND DETECTION OF DI-(2-AMINO-1-NAPHTHYL) HYDROGEN PHOSPHATE, A METABOLITE OF 2-NAPHTHYLAMINE IN DOGS*

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Troll, Belman & Nelson (1959*a*) detected a new metabolite of 2-naphthylamine in the urine of dogs. Enzymic studies indicated that this was di-(2-amino-1-naphthyl) hydrogen phosphate. They suggested that this was a potential source of 2-amino-1-naphthol, which is thought to be the active agent in the induction of bladder tumours by 2-naphthylamine. This paper describes the synthesis of the ester and its detection, by paper chromatography, in the urine of dogs dosed with 2-naphthylamine.

EXPERIMENTAL

The dogs used were beagles dosed with 2-naphthylamine five times weekly. They were kept on a diet of canned meat and dog biscuit.

Paper chromatography. Whatman no. 1 paper was employed for descending development with the solvent systems: *a*, butan-1-ol–propan-1-ol–aq. 0·1 N-NH_3 soln. (2:1:1, by vol.); *b*, butan-1-ol–acetic acid–water (2:1:1, by vol.). For the detection of compounds on paper chromatograms the reagents used were: (1) N-HCl (or 50% acetic acid) and $NaNO_2$ (0·5%) followed by hexylresorcinol (0·5% in 2N-NaOH); (2) perchloric acid–molybdate reagent (Hanes & Isherwood, 1949); (3) 0·1 M-$K_2Cr_2O_7$–acetic acid (1:1) followed by $AgNO_3$ (0·1 M) (Knight & Young, 1958); (4) sodium 1:2-naphthaquinone-4-sulphonate (0·02 M); (5) $TiCl_3$ (15%, w/v); (6) *p*-dimethylaminocinnamaldehyde (2 g. in 100 ml. of 6N-HCl and 100 ml. of ethanol) (Harley-Mason & Archer, 1958); (7) Na_2CO_3 (10%) followed by diazotized sulphanilic acid [1·6 ml. of $NaNO_2$ (0·5%) added to 10 ml. sulphanilic acid (0·2% in N-HCl)]. A Chromatolite lamp (Hanovia Ltd.) was used as a source of u.v. light. 2-Amino-1-naphthol was detected in solution by the green derivative which is formed on the addition of conc. aq. NH_3 soln. The derivative can be extracted with benzene, in which solvent it is mauve (Liebermann & Jacobson, 1882). This test is referred to in the text as the ammonia–benzene test.

Materials. 2-Amino-1-naphthyl dihydrogen phosphate was prepared by the oxidation of 2-naphthylamine with permonophosphoric acid (Boyland & Manson, 1957*a*). The ester has R_F values 0·15 in solvent system *a* and 0·7 in solvent system *b*. On paper chromatograms the compound gives a brownish-red colour with hexylresorcinol after diazotization with HCl and $NaNO_2$, but a mauve colour after diazotization with acetic acid and $NaNO_2$.

Di-(2-nitro-1-naphthyl) hydrogen phosphate. The method of Friedmann & Seligman (1950) was used for the synthesis of this compound. Phosphoryl chloride (2·3 ml.) was added to 2-nitro-1-naphthol (10 g.) in sodium-dried benzene (100 ml.), and the mixture was heated under reflux; dry pyridine (4·2 ml.) in dry benzene was added over 20 min. and heating continued for 60 min. The solution was allowed to cool overnight and was then filtered and the filtrate evaporated to dryness *in vacuo*. The residue was dissolved in acetone (100 ml.) and water (50 ml.), heated on a steam bath for 45 min. and allowed to cool overnight, when unchanged 2-nitro-1-naphthol crystallized. This was filtered off and the filtrate evaporated *in vacuo* until a solid began to separate. After cooling, this was collected. By successive evaporation and filtration further crops were collected until they became too oily to filter. At this point the oily material was redissolved by the addition of acetone, and pyridine (5 ml.) was added. The solution was again cooled, when more crystalline material was usually obtained. The combined crops, which consisted of a

* Part 7: Boyland & Williams (1960).

mixture of the nitronaphthol and the pyridine salt of the ester, were then extracted several times with hot ether to remove nitronaphthol, the pyridine salt remaining undissolved. After collection the compounds was recrystallized from aqueous acetone to yield (about 2·0 g.) the *pyridine salt of di-(2-nitro-1-naphthyl) hydrogen phosphate* as prisms, m.p. 192–195° (decomp.) (Found: C, 57·3; H, 4·0; N, 8·1; P, 6·25. $C_{20}H_{13}O_8N_2P,C_5H_5N$ requires C, 57·8; H, 3·5; N, 8·1; P, 6·0%).

On one occasion after the collection of the mixture of pyridine salt and 2-nitro-1-naphthol, further concentration of the filtrate gave the free acid. Recrystallization from aqueous acetone gave di-(2-nitro-1-naphthyl) hydrogen phosphate as needles, m.p. 187–189° (decomp.) (Found: C, 54·25; H, 3·0; N, 6·5; P, 7·2. $C_{20}H_{13}O_8N_2P$ requires C, 54·55; H, 3·0; N, 6·4; P, 7·05%). The acid was more soluble in acetone than was the pyridine salt. It was readily converted into the pyridine salt. The compound (either as free acid or pyridine salt) had an R_F value of 0·7 in solvent system *a* and gave a pale-yellow spot which darkened slightly in daylight. When sprayed with titanous chloride solution the spot gave a red colour with *p*-dimethylaminocinnamaldehyde. When heated for 7 min. with the perchloric acid–molybdate reagent at 85° and exposed to H_2S for a few minutes, the spot gave a rather feeble blue colour, masked by the yellow colour of the compound. When the compound was heated for 1 hr. at 100° with N-NaOH and the solution was then cooled, red crystals of sodium 2-nitro-1-naphthoxide separated, but paper chromatography showed that hydrolysis was not complete.

Di-(2-amino-1-naphthyl) hydrogen phosphate. Before it was realized that 2-amino-1-naphthol was always formed on the reduction of the nitroester and that the amino ester was stable in alkali, it was thought that the presence of pyridine might be deleterious. Hence the pyridine salt was converted into the sodium salt before reduction. The finely powdered di-(2-nitro-1-naphthyl) hydrogen phosphate (0·67 g.) was triturated with N-NaOH (1·3 ml.) and water (5 ml.). Pyridine and water were removed in a desiccator over H_2SO_4 and the residue was dissolved in ethanol (25%, 25 ml.). The solution was shaken with Adams catalyst (100 mg.) and H_2 at room temperature until reduction was complete (about 2 hr.). The solution was filtered, when it darkened considerably, and 2N-NaOH was added until the pH was about 9·0. The solution was evaporated almost to dryness *in vacuo* at 40–50°. The sodium salt of the amino compound separated out when almost all the water was removed. Ethanol (3 ml.) was added and the product filtered off and washed with a little cold ethanol. Recrystallization from ethanol gave *di-(2-amino-1-naphthyl) sodium phosphate trihydrate* as prisms (90 mg.), m.p. 182–184° (decomp.) (Found: C, 53·3; H, 4·9; N, 5·8; P, 6·8; loss at 100° *in vacuo*, 11·5. $C_{20}H_{16}O_4N_2PNa,3H_2O$ requires C, 52·6; H, 4·9; N, 6·1; P, 6·8; H_2O, 11·8%). The compound was soluble in acetone, water and hot ethanol. The sodium salt was precipitated from aqueous solution by the addition of NaOH. Acidification of an aqueous solution with dilute HCl gave needles. The compound was not hydrolysed by heating in N-NaOH solution but was hydrolysed by heating with 2N-HCl, when 2-amino-1-naphthol was detected. At room temperature the compound was not appreciably hydrolysed by 2N-HCl in 16 hr. The compound had an R_F value of 0·6 in solvent system *a*. The spot became pink on exposure to daylight. It gave a red colour on spraying with *p*-dimethylaminocinnamaldehyde and a pink colour with 1:2-naphthaquinone-4-sulphonic acid. It gave a yellow colour against a brown background with the $K_2Cr_2O_7$–$AgNO_3$ reagent (devised originally for divalent sulphur compounds). When sprayed with the perchloric acid–molybdate reagent and heated at 85° it gave within a few seconds a bright-blue colour. Hence it is not possible to say whether it hydrolysed under the conditions of the test to give phosphate ion which gives a blue colour only after exposure to a reducing agent, e.g. H_2S. 2-Amino-1-naphthyl hydrogen sulphate also gives a blue colour with the perchloric acid–molybdate reagent. When sprayed with $NaNO_2$ and HCl followed by hexylresorcinol a feeble mauve colour was produced. When 50% acetic acid was used in place of HCl the mauve colour with hexylresorcinol was more intense. This method of diazotization was used for detection of the metabolite. When the compound was run in solvent system *b* it gave two fluorescent spots, a major one at R_F 0·7 and a smaller one at R_F 0·9 together with a reddish-brown spot on the solvent front. The major spot gave a reddish-brown colour with hexylresorcinol after diazotization with HCl and $NaNO_2$, and a mauve colour after diazotization with $NaNO_2$ and acetic acid. These reactions were those of 2-amino-1-naphthyl dihydrogen phosphate. If, after drying the paper, it is not completely free of acetic acid, the colour after $NaNO_2$–HCl diazotization was sometimes mauve. The smaller spot gave a very feeble reaction for the di-ester. It was not always present and could sometimes be detected only by the fluorescence. The brown spot at the solvent front was due to oxidation products of 2-amino-1-naphthol, which cannot be run on chromatograms except in the presence of a reducing agent (Boyland & Manson, 1958). (If the di-ester is dissolved in solvent system *b* at room temperature and set aside for 60 min. the aminonaphthol can be detected by the ammonia–benzene test.) Phosphate ion was detected at R_F 0·4 with the perchloric acid–molybdate reagent. The decomposition of the di-ester in the butanol–acetic acid–water solvent was also observed by two-dimensional chromatography with solvent system *b* followed by *a*. The major spot then moved to R_F 0·16 in *a*, identical with the R_F of the mono-ester in this solvent system. Hence in our examination of urines and urine extracts for di-(2-amino-1-naphthyl) hydrogen phosphate by two-dimensional chromatography in solvent system *a* followed by *b*, the spot detected was the mono-ester.

2-Amino-1-naphthyl dihydrogen phosphate and 2-amino-1-naphthol (detected respectively by paper chromatography and the ammonia–benzene test) were always formed during the reduction of the nitro ester. Attempts to isolate the di-ester by dilution of the reduction liquors with water followed by acidification with acetic acid or HCl were unsuccessful, as the product was contaminated with the mono-ester and oxidation products of 2-amino-1-naphthol. The nitro ester was reduced at room temperature with $SnCl_2$ and HCl, and on heating with $FeSO_4$ and $BaCO_3$, but neither method proved a successful means of preparation of the amino ester.

Di-(2-acetamido-1-naphthyl) hydrogen phosphate. The pyridine salt (1·5 g.) of the nitro ester was dissolved in acetic acid (20 ml.). Acetic anhydride (3 ml.) was added and the solution shaken with H_2 and Adams catalyst (100 mg.) at room temperature until the reduction was complete. The solution was poured into water (100 ml.)

and acidified with conc. HCl. A gelatinous precipitate formed (this becomes more crystalline if a crystal of the compound is added to it) and was kept overnight. The product was filtered off, washed with water and crystallized from acetone to yield *di-(2-acetamido-1-naphthyl) hydrogen phosphate* (650 mg.) as plates, m.p. 160–163° (Found: C, 61·5; H, 5·2; N, 5·75; P, 6·3. $C_{24}H_{21}O_6N_2P$ requires C, 62·05; H, 4·6; N, 6·0; P, 6·7%). The ester was soluble in ethanol and hot acetone. It had R_F 0·8 in solvent system *a* and showed as a dark absorbent spot under u.v. light. In solvent system *b* it had R_F 0·87 and did not hydrolyse during chromatography as did the amino ester. Like the amino compound it gave a bright-blue colour on heating with the perchloric acid–molybdate reagent even before exposure to H_2S. After being sprayed with N-HCl and heated for 30 min. at 70° the spot reacted with *p*-dimethylaminocinnamaldehyde to give a red colour. After hydrolysis the spot also gave a blue colour with ammonia vapour due to the 2-amino-1-naphthol formed. The hydrolysed spot gave a reddish-brown colour with hexylresorcinol after diazotization with HCl and $NaNO_2$. After the ester had been warmed for a few minutes with 2N-NaOH, paper chromatography in solvent system *a* revealed the presence of 2-acetamido-1-naphthol at R_F 0·95, identified by the red colour given by diazotized sulphanilic acid or by the blue colour given when the spot was treated with HNO_2. Another product, 2-acetamido-1-naphthyl dihydrogen phosphate, was detected at R_F 0·2 as a dark absorbent spot. Hence, unlike the amino ester, acetamido ester was hydrolysed by alkali.

Paper chromatography of dog urines

The urinary bladder of a dog dosed with 2-naphthylamine was emptied by catheterization. The dog was then given 2-naphthylamine (200 mg.) by mouth, followed by water (100 ml.). After 45 min., the bladder was emptied and thereafter at 30 min. intervals until 6¼ hr. after the time of dosing. The samples were kept at 0° until examined by paper chromatography the same day with solvent system *a*; 0·025 ml. of urine was used. 2-Amino-1-naphthyl hydrogen sulphate was the only detectable metabolite in the first two specimens of urine (8 and 20 ml. respectively). Succeeding specimens (9, 5, 3, 5, 6 and 2 ml.) contained also 2-naphthylamine, 2-amino-1-naphthyl glucosiduronic acid, 2-amino-1-naphthyl hydrogen sulphate and 2-amino-6-naphthyl glucosiduronic acid. These metabolites were identified by their R_F values and colour reactions (Booth, Boyland & Manson, 1955; Boyland & Manson, 1957*b*; Boyland & Manson, 1958). Troll, Belman, Nelson, Levitz & Twombley (1959*b*) have shown that 2-amino-1-naphthyl glucosiduronic acid is a metabolite of 2-naphthylamine in dogs.

Five other metabolites were present which gave mauve colours with hexylresorcinol after diazotization with $NaNO_2$ and HCl or acetic acid. On a chromatogram in which the R_F of 2-amino-1-naphthyl hydrogen sulphate was R_F 0·5, these metabolites had R_F values of 0·42, 0·34, 0·2, 0·13 and 0·07 in solvent system *a*. Only the last one was detectable when a sample of urine which had been acidified with HCl was kept for 1 hr. at room temperature and then run in solvent system *a*. When a sample of urine was made alkaline with aq. NH_3 soln. (sp.gr. 0·88), kept for 1 hr. and re-examined, these metabolites were still present. On two-dimensional chromatograms developed with solvent system *a* and then *b* all the spots except the one with R_F 0·07 in solvent *a* lay in line with 2-amino-1-naphthyl hydrogen sulphate at R_F 0·7 after the second development. The very slow-running spot had an R_F value of 0·42 in solvent system *b*. It is probable that the compounds which can no longer be detected after acidification of the urine and which appear to yield 2-amino-1-naphthyl hydrogen sulphate in solvent system *b* are labile conjugates of the amino group of this ester (cf. Boyland, Manson & Orr, 1957).

In the fifth (2¼ hr.) and subsequent specimen of the urine a fluorescent compound was present with an R_F value of 0·6 which gave an immediate colour with *p*-dimethylaminocinnamaldehyde and a mauve colour with hexylresorcinol after diazotization with $NaNO_2$ and acetic acid. After diazotization with $NaNO_2$ and HCl only a feeble colour was given. The urines containing the metabolite were combined, adjusted to pH 4·0 and continuously extracted with ether for 3 hr. After removal of the ether the residue was examined by two-dimensional chromatography with solvent system *a* followed by *b*. The spots were treated with NH_3 vapour before running. A fluorescent compound was located which was identical in colour reaction with 2-amino-1-naphthyl dihydrogen phosphate. It had the same R_F values as the di-ester in solvent system *a* and appeared to be hydrolysed in the solvent system *b*. A mixture of the synthetic di-ester and the ether residue gave a single spot on paper chromatograms. 2-Naphthylamine, a trace of 2-amino-1-naphthyl hydrogen sulphate and 2-amino-1-naphthyl glucosiduronic acid were also present on chromatograms of the urine extract, together with another metabolite. This had the same R_F value as the sulphuric ester in the first solvent system but separated from it in solvent *b* with R_F 0·93. This gave a yellow colour with hexylresorcinol after diazotization and a positive reaction to the $K_2Cr_2O_7$–$AgNO_3$ reagent. This is believed to be an aminonaphthyl mercapturic acid (unpublished work).

The di-ester was also detected in the urine of two dogs after subcutaneous injection of 2-naphthylamine (180 mg. in 5 ml. of arachis oil), being present in the catheter specimen from 3 to 6 hr. after administration. After the injection of 2-naphthylamine into two dogs which had not previously received the amine, the di-ester was not

found in the urine. This is probably of no significance as the compound could not always be detected in the urine of continuously dosed dogs even when the dose was increased to 400 mg. Di-(2-amino-1-naphthyl) hydrogen phosphate could not be detected in 24 hr. specimens of urine or ether extracts of these. These urines, however, were not collected under sterile conditions and probably had some faecal contamination.

Troll *et al.* (1959*a*) infer that the metabolite is alkali-labile because of the appearance of free 2-amino-1-naphthol in the urine after it was made or became alkaline. Treatment of the urine samples and urine extracts which contained di-(2-amino-1-naphthyl) hydrogen phosphate with alkali did not cause any noticeable loss of the metabolite when chromatograms before and after treatment were compared. The synthetic ester is also stable to alkali. In addition, with the ether extract residue, no hydrolysis to 2-amino-1-naphthol was detected. Nevertheless, when some of the urine samples (2 ml.) obtained by catheter were made alkaline with ammonia (0·1 ml., sp.gr. 0·88), and shaken with benzene (0·3 ml.), the benzene became mauve after about 3 hr. at room temperature. In an experiment in which eight dogs were used and the urine was collected by catheter 3 hr. after dosing, no di-ester was detected in any of the samples or in ether extracts of these, but three of the urines gave positive ammonia–benzene tests.

Incubation of di-(2-amino-1-naphthyl) hydrogen phosphate with dog urine. Three samples of combined 24 hr. specimens from four dogs that had been dosed with 2-naphthylamine were adjusted to pH 5·0, 7·0 and 8·5 respectively. Di-(2-amino-1-naphthyl) hydrogen phosphate could not be detected in the samples. The di-ester (3 mg.) was added to 1 ml. samples of the urine at each pH and incubated for 16 hr. at 37°. 2-Amino-1-naphthol was not detected after 4 hr. or at the end of the incubation and chromatograms of the urine did not indicate any loss of the ester.

DISCUSSION

Examination by paper chromatography of the urine of dogs dosed with 2-naphthylamine has shown a metabolite with the properties of di-(2-amino-1-naphthyl) hydrogen phosphate to be present. This confirms the findings of Troll *et al.* (1959*a*). However, neither the metabolite nor the synthetic compound appears to be alkali-labile. Our results seem to indicate that the appearance of 2-amino-1-naphthol in alkaline dog urines may be due to the hydrolysis of another metabolite.

This new metabolite is remarkable in that the phenol is conjugated as a phosphoric ester and in that two phenolic residues are joined in the compound. Although the mammalian body contains much more phosphate than sulphate, the latter is more commonly used for conjugation and excretion of foreign phenols. The present authors had examined urine of animals treated with 2-naphthylamine specifically for 2-amino-1-naphthyl phosphate some years ago and failed to detect it.

Troll *et al.* (1959*a*) suggested that di-(2-amino-1-naphthyl) hydrogen phosphate is a probable cause of bladder cancer, because it is excreted by dogs which develop bladder cancer, and not by rabbits, in which species 2-naphthylamine does not induce bladder cancer. The compound is unusual in being soluble in ether and presumably in the cell lipids so that it might penetrate the bladder membrane and be hydrolysed to the immediate carcinogen in the bladder mucosa. This hypothesis needs further investigation but it appears to provide a possible explanation of the mechanism of the carcinogenic action of 2-naphthylamine.

SUMMARY

1. Di(2-amino-1-naphthyl) hydrogen phosphate has been synthesized by catalytic reduction of di(2-nitro-1-naphthyl) hydrogen phosphate.

2. The compound has been detected in the urine of dogs dosed with 2-naphthylamine.

3. The dog urine also contains 2-amino-1-naphthyl hydrogen sulphate, 2-naphthylamine, 2-amino-1-naphthyl glucosiduronic acid, 2-amino-6-naphthyl hydrogen sulphate and 2-amino-6-naphthyl glucosiduronic acid.

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Chromatographic Separation of Brain Lipids

CEREBROSIDE AND SULPHATIDE

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In recent years chromatography on silicic acid columns has been widely used for the resolution of the lipid mixtures extractable from living cells [egg yolk (Lea, Rhodes & Stoll, 1955; Rhodes & Lea, 1957); liver (Hanahan, Dittmer & Warashina, 1957); plasma (Garton & Duncan, 1957); spermatozoa (Lovern, Olley, Hartree & Mann, 1957); heart (Gray & Macfarlane, 1958; Gray, 1958)]. Alumina columns have also been employed in some cases [yeast (Hanahan & Jayko, 1952); egg yolk (Rhodes & Lea, 1957)]. The lipids of brain and spinal cord, which contain both cerebroside and sulphatide, have not been investigated by chromatography on alumina, and silicic acid chromatography has been applied only to enriched material, after the removal of the bulk of the other lipids by solvent fractionation (Weiss, 1956; Payne & Platt, 1958).

The purpose of the present work was to examine the chromatographic behaviour of the cerebroside and sulphatide, present in the lipid mixture extractable by chloroform–methanol from rat brain, with both alumina and silicic acid columns. Some of the early findings have already been reported in preliminary form (Long & Staples, 1959*a*).

EXPERIMENTAL

Materials

Lipids. Partially purified samples of ox-brain cerebroside (prepared by Dr R. Rodnight, Institute of Psychiatry, Maudsley Hospital, London) and of ox-brain sulphatide (Lees, Folch, Sloane-Stanley & Carr, 1959) were given by Dr G. H. Sloane-Stanley of this Department. The cerebroside sample was free from sulphatide; the sulphatide specimen had a sulphate : galactose molar ratio of 0·89. Lecithin and phosphatidylethanolamine were obtained from egg yolk by the method of Rhodes & Lea (1957). Lysolecithin was prepared according to Long & Penny (1957). Samples of phosphatidylserine and sphingomyelin were given by Dr J. N. Hawthorne and Dr June Olley respectively. Synthetic dihydrocerebroside (palmitoyl) was a gift from Dr D. Shapiro, Weizmann Institute, Rehovoth, Israel.

Solvents. Methanol was refluxed for 30 min. with NaOH pellets and granulated zinc, and distilled through a fractionating column. Chloroform was shaken five times with water, and the lower phase was distilled through a fractionating column; the water present distilled over first as a $CHCl_3$–water azeotrope and was discarded. The freshly distilled $CHCl_3$ was immediately treated with methanol, to give $CHCl_3$–methanol (98:2, v/v).

Chromatographic materials. Silicic acid (A.R. 100-mesh powder, Mallinckrodt Chemical Works; purchased from Bell and Croyden Ltd., 50 Wigmore Street, London, W. 1) was sieved and the material passing a 240 British Standards sieve was discarded. The silicic acid was then activated by heating at 120° for 24 hr. Alumina (Brockmann, grade II) was obtained from Savory and Moore Ltd., London. Two batches were used, *A* and *B*; these appeared to be similar in chromatographic properties, except that type *B* had a greater adsorptive affinity for sulphatide. The cellulose powder used was Whatman no. 1.

All other chemicals were the best available commercially.

Methods

Extracts of rat-brain lipid. The whole brains of one to four adult Wistar albino rats, of either sex, were homogenized with $CHCl_3$–methanol (2:1, v/v), 19 ml. of solvent/g. of brain being used. The homogenate was filtered through a sintered-glass funnel (porosity 2), and the residue was washed with 5 ml. of solvent/g. of brain. The combined filtrates were freed from water-soluble substances by solvent partition with 0·1 M-KCl, according to the method of Folch, Lees & Sloane-Stanley (1957), as described for ox

brain by Long & Staples (1959*b*). The $CHCl_3$-rich phase was evaporated to dryness under reduced pressure, and the residue was dissolved in $CHCl_3$–methanol–water (64:32:4, by vol.) and again evaporated to dryness. This process was repeated once more in order to decompose the proteolipid (Folch & Lees, 1951). The residue was finally suspended in $CHCl_3$–methanol (50:50 or 98:2, v/v). Protein was removed by filtration through a sintered-glass funnel (porosity 3). The filtrate, diluted to 25 ml., was stored overnight at 2° and again filtered to give a clear solution of brain lipids.

Chromatography. Alumina (13 g.) or silicic acid (7 g.) was slurried with $CHCl_3$–methanol (50:50 or 98:2, v/v, respectively) and columns (18 cm. × 1 cm. diam.) were prepared in the usual way. The lipid solution, in the appropriate solvent, was pipetted on to the column.

For gradient elution, the mixing vessel had a volume of either 125 or 200 ml., and was fitted with a mechanical stirrer. The reservoir contained the second solvent and this was arranged to run into the mixing vessel at such a rate that the volume of eluting solvent in the latter remained constant. The composition of solvent running on to the column was calculated from the exponential equation of Drake (1955). Fractions (15 or 25 ml.) were collected and transferred to glass-stoppered reagent bottles.

Analytical procedures

Galactose. The lipid solution, containing not more than 0·3 μmole of galactose, was pipetted into a 10 ml. tapered graduated centrifuge tube, and evaporated to dryness on a warm-water bath in a current of air or N_2. The residue was dissolved with warming in a mixture of ethanol (0·5 ml.) and water (0·1 ml.); 3 N-H_2SO_4 (2 ml.) was then added. At this stage most lipids, including cerebroside, produce an opalescence, but sulphatide remains in solution. The tube was covered with a glass bulb and hydrolysis was carried out for 2 hr. on a boiling-water bath (Svennerholm, 1956). After cooling, the hydrolysate was partially neutralized by addition of 11 N-NaOH (0·5 ml.). The tube was centrifuged briefly to detach water droplets from the upper part, and water was added to give a total volume of 3 ml. Powdered $BaCO_3$ (about 30–40 mg.) was then added, and the contents of the tube were well mixed and allowed to stand overnight.

After centrifuging down the $BaSO_4$ and excess of $BaCO_3$, a 2 ml. sample of clear supernatant was pipetted into a test tube (125 mm. × 16 mm. diam.) and 0·5 ml. of water was added. The tube was cooled in ice–water and 5 ml. of conc. H_2SO_4, containing 0·2% of orcinol, was added. After the contents were mixed with a glass plunger, the tube was heated on a boiling-water bath for exactly 2 min. (Brückner, 1955). The extinction of the cooled solution was read in a photoelectric colorimeter (Evans Electroselenium Ltd.), with the 624 Ilford filter (maximum transmission at 520 mμ). The same procedure was applied to duplicate galactose standards (0·3 μmole) and a water blank.

When 0·3 μmole of various lipids was tested by this procedure, the galactose value was zero for cholesterol, cholesteryl oleate, ovolecithin, phosphatidylserine, sphingomyelin and for lauric acid, myristic acid, palmitic acid, stearic acid, oleic acid and behenic acid. A mixture of phosphatidylethanolamine and ethanolamine plasmalogen (0·3 μmole), however, gave a final extinction equal to that obtainable from 0·02 μmole of galactose. Thus it was necessary to apply a small correction to galactose determinations on fractions in which appreciable quantities of ethanolamine-containing lipids were also present.

Sulphate. The lipid solution, containing not more than about 0·3 μmole of sulphatide, was evaporated to dryness in a 3 ml. tapered centrifuge tube. To the residue water (0·15 ml.) and conc. HCl (0·15 ml.) were added and the tube, capped by a glass bulb, was heated on a boiling-water bath for 1 hr. To the cooled hydrolysate 1·5 ml. of $CHCl_3$–methanol (2:1, v/v) was added and the contents were mixed thoroughly with a Pasteur pipette. The lower $CHCl_3$-rich phase, containing fatty acids, long-chain aldehydes, sphingosine and cholesterol, was withdrawn and discarded. The aqueous methanolic phase was washed twice in the same way with 1 ml. portions of $CHCl_3$–methanol (17:3, v/v). The final aqueous acidic methanolic phase was then evaporated to dryness in a current of air or N_2 on a warm-water bath. The above procedure is a modification of the method used by Lees & Folch (1959).

The residue was dissolved in water (0·15 ml.) and a solution in 0·1 N-HCl of 4-amino-4′-chlorodiphenyl hydrochloride (0·224%, containing 0·1% of cetyltrimethylammonium bromide; 0·15 ml.) was added. The tube was allowed to stand at room temperature for not less than 2 hr. (Jones & Letham, 1956). The microcrystalline ppt. of the amine sulphate was centrifuged down, and 0·1 ml. of the clear supernatant was diluted with 10 ml. of water before determination of the extinction at 254 mμ in a Unicam SP. 500 spectrophotometer. Potassium sulphate standards (0·3 and 0·15 μmole) and duplicate water blanks were subjected to the same procedure.

The 4-amino-4′-chlorodiphenyl hydrochloride was prepared according to Gelmo (1906). It has been reported by Jones & Letham (1956) that orthophosphate reacts with the reagent, but we have observed no interference from 2 μmoles of orthophosphate under the conditions used. Glycerophosphate, derived from 2 μmoles of egg phosphatidylethanolamine, also did not interfere with the determination of sulphate.

Total nitrogen. The lipid solution, containing 0·2–1·0 μg.atom of total N, was evaporated to dryness in a test tube and 0·12 ml. of 72% perchloric acid was added. Ashing was conducted on a Gallenkamp micro-Kjeldahl digestion apparatus at 170° for 2 hr. Water (2·5 ml.) was added followed by Nessler's reagent (King & Wootton, 1956; 1·5 ml.). The extinction was read in a photoelectric colorimeter (Evans Electroselenium Ltd.) with Ilford filter no. 621. Duplicate standards (1 μmole of $NH_4H_2PO_4$) and a water blank were run through the same procedure.

Sphingosine. The lipid solution, containing not more than 1 μmole of sphingosine, was evaporated to dryness; the residue was hydrolysed by heating for 1 hr. on a boiling-water bath with 0·5 ml. of 6 N-HCl (Robins, Lowry, Eydt & McCaman, 1956). The cooled hydrolysate was treated with 2·5 ml. of $CHCl_3$–methanol (2:1, v/v) and, after being mixed thoroughly with a Pasteur pipette, the lower $CHCl_3$-rich phase was transferred into a second tube. The aqueous methanolic phase was extracted twice more with 1 ml. portions of $CHCl_3$–methanol (17:3, v/v). The combined $CHCl_3$-rich extracts were evaporated to dryness and ashed for total N, as already described.

Amino nitrogen. The lipid sample, containing not more than about 0·2 μg.atom of amino N, was evaporated to dryness, treated with 0·1 ml. of water and 2·0 ml. of the ninhydrin reagent of Moore & Stein (1948), and heated on

a boiling-water bath for 20 min. After dilution of the solution to 10 ml. with ethanol–water (80:20, v/v), the extinction was determined in a photoelectric colorimeter (Evans Electroselenium Ltd.) with Ilford filter no. 626. Standard $NH_4H_2PO_4$ (0·2 μmole) and a water blank were simultaneously run through the same procedure.

Total phosphorus. The lipid sample, containing not more than about 0·2 μg.atom of total P, was evaporated to dryness and ashed with 0·25 ml. of 72% perchloric acid at 170° for 2 hr. After dilution with water to 2 ml., the tube was placed in a boiling-water bath for 20 min. The cooled solution was analysed for orthophosphate by the method of Berenblum & Chain (1938), as modified by Long (1943).

Acyl ester. Determinations were made according to Shapiro (1953).

Cholesterol. This was determined by the Liebermann–Burchard procedure (King, 1946).

RESULTS

Removal of water-soluble contaminants and proteolipid protein from lipid extracts of rat brain

Since the aim of the present work was to study the chromatographic behaviour of cerebroside and sulphatide, it was necessary to ensure that the lipid extracts under examination were free from other carbohydrate-containing substances. The crude lipids prepared by extraction of brain tissue with $CHCl_3$–methanol (2:1, v/v) are well known to contain ganglioside (Folch, Arsove & Meath, 1951), and may contain traces of free monosaccharides and their phosphate esters. These water-soluble materials may be removed by solvent partition (Folch *et al.* 1957; Long & Staples, 1959*b*). As further experiments were planned to study the chromatographic behaviour of the ethanolamine- and serine-containing phospholipids, it was also considered desirable to remove the proteolipid protein. The crude lipid extract of brain (stage 1) was therefore subjected to the following successive treatments: (*a*) removal of water-soluble components by solvent partition (stage 2); (*b*) precipitation of proteolipid protein by heat treatment (stage 3); (*c*) passage through a cellulose column (stage 4), according to Lea & Rhodes (1953). At each stage, analyses were made for galactose, sphingosine, total N, amino N, total P, ester and cholesterol. The results, shown in Fig. 1, are in terms of μmoles or μg.atoms of constituent/g. of fresh rat brain.

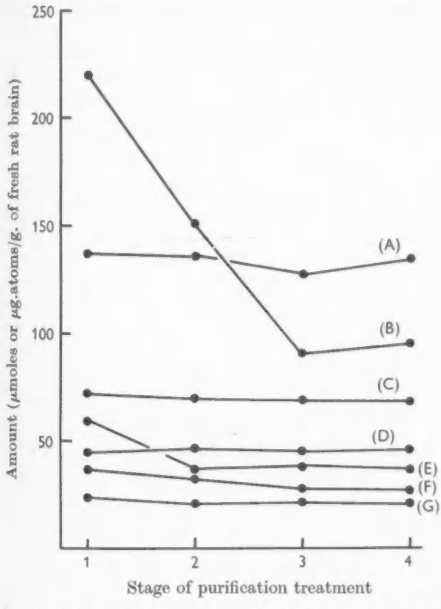

Fig. 1. Analytical values, in μmoles or μg.atoms/g. of fresh tissue, for a $CHCl_3$–methanol (2:1, v/v) extract of rat brain (stage 1), and after the following successive treatments: washing out of water-soluble substances (stage 2), removal of proteolipid protein (stage 3) and passage through a cellulose column (stage 4). (A) Acyl ester; (B) total N; (C) total P; (D) cholesterol; (E) amino N; (F) sphingosine; (G) galactose.

It will be seen from Fig. 1 that the cholesterol remains constant throughout the series of treatments. Thus analyses for this typical lipid constituent indicated that no manipulative losses occurred. Acyl ester and total P contents also varied very little, as would be expected. Total N and amino N fell significantly after the solvent-partition procedure (stage 2), reflecting the loss of free amino acids, and total N showed a further substantial decrease after removal of proteolipid protein (stage 3). There was a slight fall in galactose and sphingosine contents after the solvent-partition process, which largely represents the removal of ganglioside. There also seems to be a further slight fall in sphingosine after removal of proteolipid protein. However, this may be due to a slight solubility in the $CHCl_3$-rich phase of the less polar amino acids formed on hydrolysis of the proteolipid protein in stage 2; such behaviour would have contributed to the apparent sphingosine content.

Passage through a cellulose column (stage 4) did not lead to the removal of any further components for which analyses were made. This suggests that the solvent-partition procedure is adequate to remove water-soluble material. Stage 4 was omitted in subsequent experiments.

Chromatography on alumina

Samples of the cerebroside and sulphatide preparations were chromatographed on alumina in the following way. The material was dissolved in $CHCl_3$–methanol (80:20, v/v) and 2 ml. of the

solution was applied to an alumina column, equilibrated with the same solvent. Type *A* alumina was used. The column was washed with 25 ml. of $CHCl_3$–methanol (80:20, v/v) followed by 50 ml. of $CHCl_3$–methanol (50:50, v/v), the eluate being collected in 25 ml. fractions. Gradient elution was then applied. The mixing vessel contained 125 ml. of $CHCl_3$–methanol (50:50, v/v), and the reservoir 150 ml. of $CHCl_3$–methanol–water (45:45:10, by vol.). Eleven more 25 ml. fractions were collected and galactose was determined on each fraction. The results are shown in Fig. 2.

It will be observed that the peak derived from the cerebroside sample occurred in fraction 8, whereas that from the sulphatide was in fraction 11. The curve for the cerebroside sample also exhibited a shoulder in fraction 11, although the material was free from sulphatide.

Similar types of experiments with type *A* alumina were conducted on the washed deproteinized lipids of rat brain (stage 3 of the previous section), except that the mixed lipids were loaded on to the column in $CHCl_3$–methanol (50:50, v/v). The results are shown in Fig. 3.

The fractions collected before gradient elution was begun contained all the cholesterol of the lipid applied to the column, as well as the choline-containing phospholipids (Rhodes & Lea, 1957). When gradient elution was started, galactose-containing fractions were eluted to give a curve which was similar in appearance to that obtained for the cerebroside sample shown in Fig. 2. The galactose peak appeared in fraction 8, but the shoulder was rather more prominent. In this experiment, the recovery of galactose in the seven fractions (nos. 6–12) was 22·3 μmoles/g. of brain, compared with 21·3 μmoles applied to the column. Thus both cerebroside and sulphatide must have been completely eluted, the latter probably contributing to the shoulder (fraction 10). Total N and sphingosine analyses were also carried out on the combined galactose-containing fractions, and the molar ratio (total N:sphingosine N:galactose) was 1·00:1·01:1·01. These fractions usually contained a trace of acyl ester, unaccompanied by either phosphorus or amino N; it is thought that this acyl ester is derived from the breakdown of aminophospholipids, which occurs on alumina columns (Long & Staples, 1960; Long, Shapiro & Staples, 1960).

In fraction 13, aminophospholipids began to be eluted, as indicated by determinations of amino N, phosphorus and ester; this material has been shown to be ethanolamine-containing lipid (Long & Staples, 1960). However, these amino-containing fractions are well separated from those which contain galactose. It is clear that the order of elution is: cerebroside, sulphatide, ethanolamine-

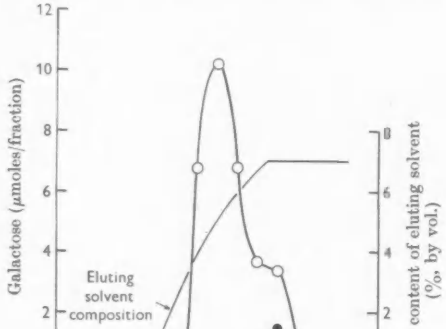

Fig. 2. Chromatographic behaviour of samples of cerebroside (45·7 mg.; ○) and sulphatide (5·20 mg.; ●) on an alumina (type *A*) column. The lipids were applied to the column in $CHCl_3$–methanol (80:20, v/v). The solvent was then changed to $CHCl_3$–methanol (50:50, v/v), and this was followed by gradient elution (composition shown), whereby the water content of the latter solvent was increased from 0 to 7% (by vol.). Fractions, 25 ml.

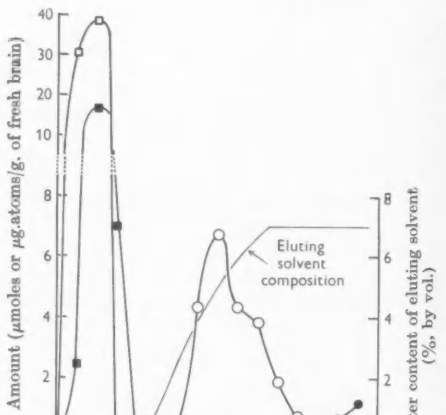
Amount (μmoles or μg.atoms/g. of fresh brain)
Water content of eluting solvent (%, by vol.)
Eluting solvent composition
Fraction no.

Fig. 3. Chromatography of washed, deproteinized, rat-brain lipids on alumina (type *A*). The lipids were applied to the column in $CHCl_3$–methanol (50:50, v/v); other conditions were as in Fig. 2. Cholesterol (□), total phosphorus (■), galactose (○), amino nitrogen (●). Composition of eluting solvent is shown.

containing phospholipid. These experiments were carried out before a reliable method of determining sulphate had been established.

When the sample of type *A* alumina, which was used in the foregoing work, had been used up, subsequent samples (designated type *B*) were found to possess the same affinity for cerebroside and ethanolamine-containing phospholipid, but to be rather more retentive with respect to sulphatide. In these experiments, which were carried out exactly as previously described, sulphate determinations were also made. A typical result is shown in Fig. 4.

After the application of gradient elution, galactose and sphingosine contents were in close agreement, the main peak again being found in fraction 8. Sulphatide, which began to be eluted in fraction 10, reached its peak in fraction 12, and beyond this point, sulphate, galactose and sphingosine values were almost identical, indicating that sulphatide free from cerebroside was being eluted. If the cerebroside content is taken to be equal to the difference between galactose and sulphate, the trailing edge of its elution curve is shown by the discontinuous line in Fig. 4. From this, it will be seen that with alumina type *B*, cerebroside and aminophospholipid are completely separated, whereas sulphatide overlaps the aminophospholipid as well as the cerebroside. The order of elution is again: cerebroside, sulphatide, aminophospholipid.

Chromatography on silicic acid

In these experiments, a sample of the washed, deproteinized, mixed lipids of rat brain (stage 3), dissolved in $CHCl_3$–methanol (98:2, v/v), was loaded on to a silicic acid column, equilibrated with the same solvent. After washing in the lipid, gradient elution was applied. The mixing vessel contained 200 ml. of $CHCl_3$–methanol (98:2, v/v), with 200 ml. of $CHCl_3$–methanol (90:10,v/v) in the reservoir; 15 ml. fractions were collected (Fig. 5). The cholesterol was completely eluted in the first three fractions. It was accompanied by a trace of ester, but was free from nitrogen and phosphorus. Immediately afterwards, a small galactose-containing peak was eluted (fractions 4–7); this accounted for 5% of the total galactose applied to the column. The major peak followed in fractions 10–18, representing 82% of the galactose applied. Both these peaks were free from sulphate and amino N. Beginning with fractions 20, however, aminophospholipid began to be eluted. After collection of 24 fractions, the eluting solvent was changed to $CHCl_3$–methanol (80:20, v/v). This first eluted a sharp peak of aminophospholipid, and later a third galactose-containing peak. In the latter, approximately equivalent amounts of galactose and sulphate were present. The galactose content accounted for 13% of the amount applied, and the sulphate was equivalent to 89% of that present in the original lipid. Thus the whole of the galactose and almost all the sulphate were recovered.

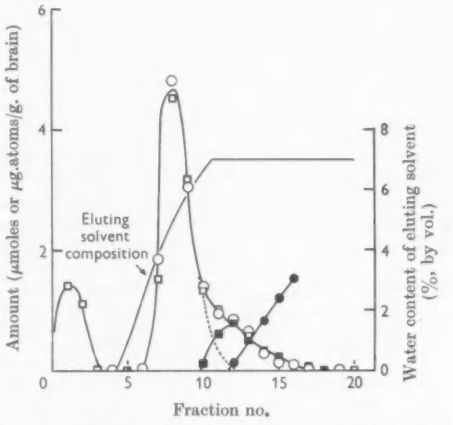

Fig. 4. Chromatography of washed, deproteinized, rat-brain lipids on alumina (type *B*). Experimental conditions were as in Fig. 3. Sphingosine (□), galactose (○), sulphate (■), amino nitrogen (●). Composition of eluting solvent is shown.

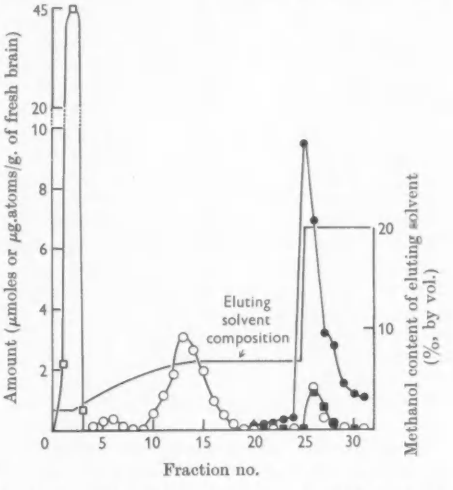
Amount (μmoles or μg.atoms/g. of fresh brain)
Methanol content of eluting solvent (%, by vol.)
Eluting solvent composition
Fraction no.

Fig. 5. Chromatography of washed, deproteinized, rat-brain lipids on silicic acid. The lipids were applied in $CHCl_3$–methanol (98:2, v/v). Cholesterol (□), galactose (○), amino nitrogen (●), sulphate (■). Composition of eluting solvent is shown.

Chromatography on silicic acid thus separated the galactose-containing lipids into three discrete peaks. The sulphatide is eluted much later than the main cerebroside peak, in contrast with its behaviour on alumina, where overlapping is observed (Fig. 4). Furthermore, the aminophospholipid precedes sulphatide on silicic acid, whereas the order on alumina is the reverse of this. In the main peak, galactose, total N and sphingosine N were equivalent to one another.

DISCUSSION

Previous investigations on the chromatographic behaviour of cerebroside and sulphatide have invariably been carried out on starting material, either brain or spinal cord, which had already been subjected to some solvent-fractionation technique in order to remove the bulk of the accompanying lipids. Such treatment normally leads to some loss of galactose-containing lipids. Radin, Brown & Lavin (1956) (see also Radin & Brown, 1960) extracted spinal-cord lipids with ether to remove cholesterol and other ether-soluble material, and chromatographed the residual lipids on Florisil, which retained the phospholipids. Sulphatide and cerebroside were then separated by adsorbing the former on an anion-exchange resin. Weiss (1956) extracted brain or spinal cord with acetone followed by light petroleum before subjecting the residual lipids to silicic acid chromatography.

In the experiments described here, lipid extracts of brain were chromatographed without previous treatment, other than removal of water-soluble substances and proteolipid protein. The results of chromatography on alumina are complicated by the slightly different properties of the two batches used, type *B* alumina having a greater affinity for sulphatide than had type *A*. It is clear that with the latter, a quantitative separation of cerebroside plus sulphatide from other brain lipids has been achieved (Fig. 3), whereas with type *B* alumina the sulphatide and ethanolamine-containing lipids overlap (Fig. 4). This observation is of some interest, since we have already noted the ion-exchange properties of alumina (Long *et al.* 1960). Different batches of alumina might therefore be expected to exhibit greatest variations with respect to electrically charged lipids. With type *B* alumina, the eluted sulphatide was found to contain no K^+ ions, as shown by flame-photometric determinations, even though the mixed lipids had earlier been washed with 0·1 M-potassium chloride. This suggests that type *B* alumina removes K^+ ions from their association with sulphatide. We have no parallel data for type *A* alumina.

It should be mentioned that the cerebroside and sulphatide components of rat brain exhibited similar chromatographic behaviour on alumina, independently of whether the proteolipid protein had been removed or not. This observation indicates that brain proteolipids (Folch & Lees, 1951) either do not contain cerebroside and sulphatide, or that the association between these lipids and protein is readily broken in contact with alumina.

Although the best separation of galactose-containing lipids from ethanolamine-containing lipids on alumina columns is achieved by the use of gradient elution, a fair separation can also be obtained by stepwise elution. Such a procedure was first developed in the early stages of this work and was later adopted by Davison, Dobbing, Morgan, Wajda & Payling-Wright (1960). However, under these conditions, we found that the early galactose-containing fractions were usually contaminated with a small amount of aminophospholipid, a feature which is absent when the gradient-elution technique is used.

The results obtained with rat-brain lipids on silicic acid columns (Fig. 5) may be compared with those reported for monkey-brain lipids by Weiss (1956), although the latter did not specify the exact composition of the chloroform–methanol mixtures used in his gradient-elution experiments. At low methanol concentrations, Weiss's peak I (containing 4·4% of the total galactose applied to the column) may be taken to correspond with our first peak (5%). His peaks II and III taken together (62·6%) may be similar to our main peak (82%), although his peak III had twice the expected content of total nitrogen; this latter finding could result from contamination with ethanolamine-containing lipid due to the use of too steep a gradient. Peak IV (19·3%) is almost certainly the same as our sulphatide peak (13%), and bears a rather similar relation to the elution pattern of phospholipid noted by us. The essential difference between the observations of Weiss (1956) and ourselves is that we do not find any separation of the main cerebroside peak into two fractions. However, this may be related to possible differences in the nature of the component fatty acid radicals, caused by the use of different animal species.

SUMMARY

1. The material extracted from rat brain by means of chloroform–methanol (2:1, v/v) has been analysed for galactose, sphingosine, total nitrogen, amino nitrogen, total phosphorus, cholesterol and acyl ester. Similar analyses were carried out on the extract after removal of water-soluble substances and proteolipid protein. The results indicate that no lipids, other than ganglioside, are lost in the course of this purification procedure.

2. Chromatography of the washed, deproteinized, lipid extract on alumina with a gradient-elution technique, in which the water content of the solvent was slowly increased, led to the successive elution of cerebroside, sulphatide and aminophospholipid. The cerebroside and sulphatide peaks always overlapped. With one batch of alumina the galactose-containing lipids were completely separated from the aminophospholipid.

3. Gradient-elution chromatography on silicic acid, whereby the methanol content of the solvent was increased, led to the complete separation of the cerebroside from the sulphatide. The former was also separated from the aminophospholipid, whereas there was overlapping between the sulphatide and aminophospholipid.

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The Phospholipids of Butter and their Effect on Blood Coagulation

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The suggestion by Macfarlane (1955) that dietary fats may play an important role in the aetiology of atheroma and that different lipids may have widely different effects on blood thromboplastin formation led us to investigate the effect of a number of dietary fats on blood coagulation *in vitro*. At the start of this work it was generally believed that the lecithin group of phospholipids were strong potentiators of blood coagulation (Macfarlane, Trevan & Attwood, 1941; Fullerton, Davie & Anastasopoulos, 1953) but later work by a number of workers showed that phosphatidylethanolamine present in the crude lecithin was the active material, and that pure lecithin was completely inactive (Poole, Robinson & Macfarlane, 1955; Lea, Rhodes & Stoll, 1955; Lea, 1956; O'Brien, 1956*a*, *b*; Robinson & Poole, 1956). Poole & Robinson (1956) have since demonstrated that synthetic specimens of

phosphatidylethanolamine were as active, in rat-plasma systems, as that isolated from eggs. On the other hand, Biggs & Bidwell (1957) in their experiments on the fractionation of brain phospholipids found the blood-clotting activity variably distributed through the fractions and their phosphatidylethanolamine fraction appeared to be the least active of those tested. They further found that synthetic dimyristoylphosphatidylethanolamine was inactive in human plasma even in the presence of egg lecithin as an emulsifier. The activity of synthetic phosphatidylethanolamine was slight in rat-plasma systems in comparison with human-brain phosphatides.

In the present work, the clotting activity of a large number of fats and lipid fractions had to be tested and hence a rapid blood-clotting technique involving a fat-free thromboplastin was required. The choice of test was therefore limited to the plasma Stypven (Russell viper venom, R.V.V.) method as used by Macfarlane *et al.* (1941), venom being one of the few thromboplastins which is free of fat. A modification of the above method, the 'instantaneous' technique (Billimoria, Lockey & Maclagan, 1957), was preferred, since with this technique the lipolytic action of venom on plasma fats was minimized, and interfering effects of lipids produced by lipolysis were thus reduced.

The effect of a number of the usual dietary fats on this system showed that the clotting effect was almost entirely confined to milk fats and was maximal in butter (Maclagan & Billimoria, 1956). The activity was associated with the lipoprotein in butter, and further separation indicated that the active materials were phospholipids. This paper describes the preliminary separation of the phospholipids from butter and the further fractionation of these lipids by continuous gradient elution from silicic acid columns. The separation was controlled throughout by tests of thromboplastic activity since we were mainly interested in fractions with this action. A preliminary account of some of the work has been published (Billimoria & Curtis, 1959).

METHODS

Total nitrogen. This was determined by nesslerization by the method of King & Wootton (1956) with a modified digestion mixture (C. Long, personal communication) consisting of 100 ml. of H_2SO_4, 25 g. of K_2SO_4, 25 mg. of HgO and water to 2 l. Digestion of the lipid containing usually 10–20 μg. of N was carried out with 0·5 ml. of the digestion mixture heated for 20 min. on an electric micro-digestion apparatus (A. Gallenkamp and Co. Ltd., London, E.C. 2).

Amino nitrogen. This was determined on the intact lipids by the procedure of Lea & Rhodes (1954); preliminary hydrolysis with 0·5 N-HCl at 100° for 15 min. was employed for the glycosides.

Phosphorus. This was determined after removal of the solvent followed by digestion with perchloric acid by a slight modification of the method of Allen (1940) in which the volume used in the colorimetric determination was decreased in order to increase the sensitivity of the method. Perchloric acid (0·5 ml., 60%, w/v) was used for the digestion of lipid containing 2–50 μg. of P. The digest was diluted with 5 ml. of water followed by the addition of 0·25 ml. of 8% (w/v) ammonium molybdate and 0·5 ml. of amidol reagent made up by dissolving 1 g. of amidol in 100 ml. of water containing 18·3 g. of sodium metabisulphite. The blue colour obtained was read after 12 min. in a Unicam SP. 600 spectrophotometer at 690 mμ in a 1 cm. cuvette.

Sugars. These were estimated by the orcinol–H_2SO_4 method cited by Winzler (1955).

Paper chromatography of bases and sugars formed on hydrolysis. For bases, a sample of the combined peak fractions, obtained by chromatography, containing about 5 mg. of the lipids was introduced into a thick-walled Pyrex tube; the solvent was evaporated *in vacuo* and 1 ml. of 6 N-HCl added. The tube was sealed and heated overnight at 100°. The tube was cooled and cut open and the HCl evaporated off *in vacuo* (0·1 mm. Hg). Water (0·5–1 ml.) was added and the solution evaporated again to remove traces of HCl. The residue was dissolved in 0·1–0·2 ml. of water, and 5 μl. of the solution was applied to the paper.

For chromatography of sugars, samples containing 5 mg. of sugar were hydrolysed for 2 hr. at 100° with 2 ml. of N-H_2SO_4. An excess of $BaCO_3$ was added, and the mixture was heated to boiling and filtered. The precipitate was washed with 5 ml. of water, and the filtrate was evaporated to dryness *in vacuo* (0·1 mm. Hg). The residue was dissolved in 0·5 ml. of water, and 5–10 μl. was applied to paper for chromatography.

For chromatography of phosphate esters, samples containing 5 mg. of phosphatide were hydrolysed with 6 N-HCl (1 ml.) at 100° for 15 min. After dilution with water (1 ml.), the solution was filtered and the precipitate washed with water (1 ml.). The filtrate and washing were evaporated to dryness at 50° *in vacuo*, and the residue was dissolved in water (0·5 ml.). Portions containing 20 μg. of phosphorus were applied to Whatman no. 54 paper and chromatographed in ethyl acetate–pyridine–water as described by Hanes & Isherwood (1949).

Preparation of silicic acid. Silicic acid (500 g., 100-mesh, Mallinckrodt A.R. grade from Savory and Moore Ltd., Lawrence Rd., London, N. 15) was thoroughly mixed with 2 l. of water and the suspension allowed to settle for 1 hr. in a 2 l. measuring cylinder. The supernatant liquid containing the finer particles was rejected, and the residue was filtered off under suction and dried in an air oven overnight at 105–110°. The material was lightly ground and sieved through a 100-mesh nylon gauze and stored in a tightly stoppered bottle.

Characterization of phospholipids. The various phospholipid fractions, obtained by column chromatography, were identified and characterized by chromatography of the unhydrolysed phospholipids on silicic acid-impregnated paper, by the methods of Marinetti, Erbland & Kochen (1957) and Lea *et al.* (1955). Rhodamine 6G followed by ultraviolet light was used to detect all lipids, the ninhydrin reaction to detect the amino lipids and phosphomolybdic

acid reagent for lipids containing choline. Sphingomyelin was also characterized by its reineckate reaction, its resistance to mild alkaline hydrolysis and the presence of a chloroform-extractable nitrogenous base after prolonged hydrolysis with $Ba(OH)_2$ solution followed by acidification. Choline was detected by the method of Levine & Chargaff (1951) and by its reineckate reaction (Glick, 1944).

After hydrolysis of the lipids, the bases liberated were characterized by paper chromatography by the methods of Datta, Dent & Harris (1950). Phosphoric esters were detected by the method of Hanes & Isherwood (1949), and glycerophosphoric acid was determined by the method of Burmaster (1946). Sugars were detected by paper chromatography (Jermyn & Isherwood, 1949).

Fractions containing sugar. The sugar was shown to be present in chemical combination as follows. Emulsification of the individual fraction with water in a sonic vibrator followed by Seitz filtration gave a filtrate free of sugar, and fractionation between water and chloroform gave an aqueous layer free of sugar. The sugars were, however, readily liberated by acid hydrolysis as indicated above.

Butter. Numerous samples of butter were used in this work, all obtained by retail purchase. No obvious differences were noted between different brands bought at various seasons of the year.

Phospholipids. Synthetic L-α-dimyristoylphosphatidylethanolamine and L-α-dimyristoyl-lecithin were kindly supplied by Dr Erich Baer (University of Toronto, Canada). A sample of sphingomyelin was kindly supplied by Dr Elizabeth Smith (Courtauld Institute of Biochemistry, Middlesex Hospital Medical School, London, W. 1) and others were purchased from L. Light and Co. Ltd., Colnbrook, Bucks, England.

Thromboplastic activity. The blood-clotting activity of the lipids was tested on normal fasting-human plasma by the Stypven method and by plasma and whole-blood recalcification techniques as described earlier (Maclagan & Billimoria, 1956; Billimoria *et al.* 1957). The thrombin- and thromboplastin-generation tests used were performed as described by Biggs & Macfarlane (1957). The weight of lipid added was obtained by multiplying its P content by 25.

Apparatus. A graduated cylindrical donor vessel diam. 60·6 mm. containing methanol (1·5 l.) was connected at the base by means of a stopcock (S_1) to a similar receiver vessel (R) diam. 83·5 mm. containing $CHCl_3$ (1·5 l.). The column containing silicic acid was connected by means of a stopcock (S_2) attached to a second outlet in (R) and the fractions were collected in a fraction collector. Vessel (R) carried a small iron nail covered in a Fluon sleeve which, in conjunction with a rotating magnet held under the receiver, acted as a stirrer.

The diameters of the vessels were such that when, with the stirrer in motion, the stopcock (S_2) was closed and (S_1) opened, there was no solvent flow from one cylinder to the other. The gradient elution was started by opening the stopcock (S_2).

The gradient obtained was checked by introducing a standard dye solution of Sudan Black in methanol in the donor vessel and reading the colour of the resulting mixture of $CHCl_3$–methanol–dye issuing from the receiver vessel and collecting in the fraction cutter tubes.

The gradient could also be obtained by a simple calculation without performing the above experiment. If H_1 is the height of the methanol and H_2 the height of the mixture after a volume V has been collected, then $H_1/H_2 = \rho_1/\rho_2$, where ρ_1 and ρ_2 are the densities of methanol and mixture respectively. The density of the mixture (ρ_2) can be found after a fixed volume V leaves the cylinder (R) from measurements of H_1 and H_2. Since the change in volume due to the mixing of $CHCl_3$ and methanol was found to be negligible, the percentage composition of the two solvents may be read off directly from a reference line drawn joining the two points corresponding to the density of pure $CHCl_3$ and pure methanol.

RESULTS

Dietary fats and Stypven times

The Stypven clotting times of plasma were substantially decreased by adding certain fats to plasma. Thus Fig. 1 shows the effect of two typical fats, indicating that butter is much more effective than margarine at all concentrations tested. The maximum effect was produced by 10 mg. of butter/system, which produced a 78% drop in clotting time.

Clotting unit. Since butter was the most active natural fat tested, the activity of the least amount of butter (10 mg.) which produced the maximum shortening of clotting time was chosen as one clotting unit. The other fats could then be assigned units/mg. With feebly active fats or certain lipid fractions available only in limited quantity, it was impossible to produce minimum clotting times and the units were read from the calibration curve based on a serial dilution of butter. Thus if 0·01 mg. of a substance gave a clotting time of 11 sec. with the plasma used in Fig. 1, corresponding to 1 mg. of butter (0·1 unit), this substance would have an activity of 10 units/mg.

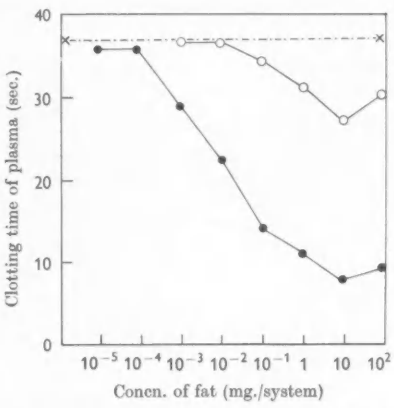

Fig. 1. Effect of adding butter or margarine on the Stypven clotting times of normal fasting plasma. ●, Butter; ○, margarine; ×, control.

Other clotting systems

The effect of various concentrations of butter and margarine on the plasma recalcification system were also studied; Fig. 2 shows that again butter was far more active than margarine. Maximum effects were obtained from 5 mg. of butter and a much smaller maximum from 1 mg. of margarine. The inhibitory effects obtained with larger quantities of the fats were quite pronounced with this clotting system, especially with margarine, which gave a clotting time longer than the control at a concentration of 10 mg./system. This latter effect is not clearly understood but may be due to mechanical interference of neutral fat with the clotting system.

Butter was also tested in the whole-blood coagulation system, and the thrombin- and thromboplastin-generation tests; it was active in all three systems.

Fractionation of butter

A typical preliminary separation of active material from butter is shown in Fig. 3, where the progress of fractionation was followed by estimation of clotting activity at various stages. Butter (1) was melted overnight at 40° and centrifuged (2000 ***g*** for 0·5 hr.), when four visible layers were formed. The top, clear, neutral-fat layer (2) was almost inactive; this was followed by three aqueous lipoprotein layers which contained the major proportion of the activity. These aqueous layers (3) were combined and extracted with ether at 40° to remove most of the residual neutral fat. In this process only 10 % of the activity was found in the extract (4), even when chloroform was used instead of ether, suggesting that the active lipid is present mostly in the form of lipoprotein at this stage. In confirmation of this, when the ether-insoluble residue (5) was treated with boiling acetone and the mixture filtered after cooling, the activity of the dry, acetone-insoluble precipitate (7) was almost quantitatively extracted by chloroform (Soxhlet extraction). The chloroform extract (9) was evaporated to a small bulk, and the phospholipids (10), precipitated at 0° with a large volume of acetone, had an activity of approx. 40 units/mg. This represented a 400-fold concentration and an overall recovery of 74 % of the total original activity. The phospholipids, dissolved in a minimum of chloroform, were adsorbed on a short silicic acid column, and the column was washed with chloroform to remove non-phospholipid fractions. The crude phospholipids (12) were eluted with ethanol–chloroform–water (6:3:1, by vol.) and, after evaporation of the solvent, had an activity of approx. 60 units/mg. The acetone-soluble fractions (6) and (11) and the residue insoluble in chloroform (8) were almost inactive.

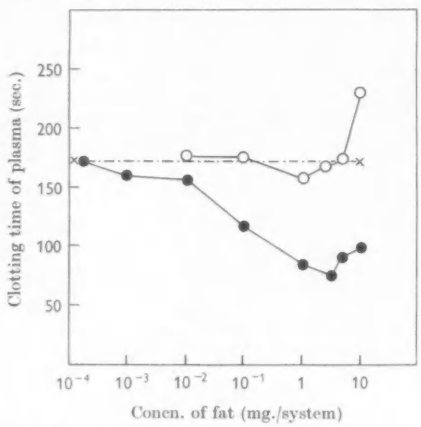

Fig. 2. Effect of adding butter or margarine on the recalcification times of normal fasting plasma. ●, Butter; ○, margarine; ×, control.

Having once established that the activity from butter was due to its phospholipid content, for further work, the chloroform extracts from stage (9) were directly adsorbed on a column of silicic acid and concentrated as indicated at stage (12). Thus, in large-scale work, extracts of as much as 15 g. of total lipid containing about 1 g. of phospholipid could be directly adsorbed on large-diameter columns (2½ in.) containing silicic acid (125 g.), and the lipids other than phospholipids eluted off the column with chloroform (usually about 2 l.). The phospholipids were then eluted off the columns with the mixture of ethanol–chloroform–water. After evaporation of this solvent, the lipid, dissolved in a small volume of dry chloroform, was adsorbed on to a second column for stepwise or gradient elutions, as described below.

Stepwise elution of butter phospholipids from a silicic acid column

The above phospholipids, dissolved in a small volume of dry chloroform, were readsorbed on a column of silicic acid (125 g.) and eluted with graded amounts of methanol, 1, 2, 4, 8, 16 and 32 % (v/v) in chloroform and finally with a mixture of ethanol–chloroform–water (6:3:1, by vol.). The separation was followed by weighing the lipids eluted and determining their clotting activity. At each change of methanol concentration a sharp peak occurred, but the analytical data on the eluates showed that most of the substances obtained were still mixtures, and consistent results were unobtainable from repeated experiments.

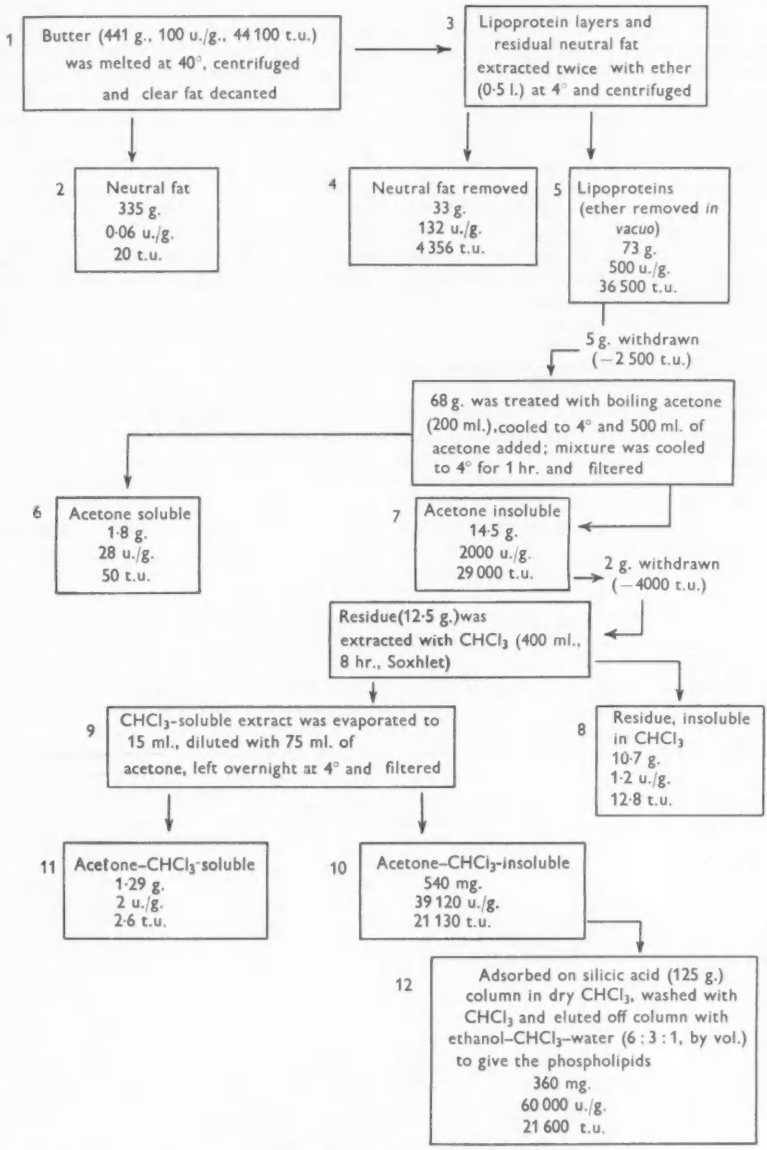
1
Butter (441 g., 100 u./g., 44 100 t.u.) was melted at 40°, centrifuged and clear fat decanted
3
Lipoprotein layers and residual neutral fat extracted twice with ether (0·5 l.) at 4° and centrifuged
2
Neutral fat
335 g.
0·06 u./g.
20 t.u.
4
Neutral fat removed
33 g.
132 u./g.
4 356 t.u.
5
Lipoproteins (ether removed in vacuo)
73 g.
500 u./g.
36 500 t.u.
5 g. withdrawn (−2 500 t.u.)
68 g. was treated with boiling acetone (200 ml.), cooled to 4° and 500 ml. of acetone added; mixture was cooled to 4° for 1 hr. and filtered
6
Acetone soluble
1·8 g.
28 u./g.
50 t.u.
7
Acetone insoluble
14·5 g.
2000 u./g.
29 000 t.u.
2 g. withdrawn (−4000 t.u.)
Residue(12·5 g.)was extracted with CHCl3 (400 ml., 8 hr., Soxhlet)
9
CHCl3-soluble extract was evaporated to 15 ml., diluted with 75 ml. of acetone, left overnight at 4° and filtered
8
Residue, insoluble in CHCl3
10·7 g.
1·2 u./g.
12·8 t.u.
11
Acetone–CHCl3-soluble
1·29 g.
2 u./g.
2·6 t.u.
10
Acetone–CHCl3-insoluble
540 mg.
39 120 u./g.
21 130 t.u.
12
Adsorbed on silicic acid (125 g.) column in dry CHCl3, washed with CHCl3 and eluted off column with ethanol–CHCl3–water (6 : 3 : 1, by vol.) to give the phospholipids
360 mg.
60 000 u./g.
21 600 t.u.

Fig. 3. Preliminary separation of phospholipids from butter. u, Stypven clotting units; t.u., total units of clotting activity.

Elementary chemical analysis of the lipids and paper chromatography of intact lipids and their hydrolysis products show that the 8% fraction contained mainly serine, glycerophosphoric acid and galactose; ethanolamine was also present to a lesser extent in the hydrolysate. The fraction had a clotting activity of approx. 200 units/mg. The analysis (Found: C, 61·6; H, 10·1; N, 1·5; P, 3·7%) was in close agreement with that of a phosphatidylserine galactoside containing C_{18} and C_{20} acids, with an empirical composition $C_{49}H_{95}O_{14}NP$ (requires C, 61·8; H, 10·0; N, 1·5; P, 3·4%).

The 32% fraction was of the lecithin type since it gave no ninhydrin reaction even after hydrolysis and contained choline, glycerophosphate, glucose and galactose. The P:N ratio was 1:1·14 and the low carbon value was due to the glycoside structure (Found: C, 57·1; H, 10·1; N, 1·9; P, 3·7%). The fraction was inactive in the clotting test.

The ethanol–chloroform–water fraction contained mainly sphingomyelin as shown by paper chromatography, precipitation reaction with Reinecke's salt, and presence of choline and absence of glycerophosphate in the hydrolysate. The P:N ratio was 1:2·14 (Found: C, 57·9; H, 10·3; N, 2·9; P, 3·0%). The 1, 2, 4 and 16% fractions appeared to be mixtures and were not further investigated.

Gradient elution chromatography of phospholipids

Further information was obtained by elution of the lipids from a silicic acid column by a linear gradient elution technique; the crude phospholipids, after removal of neutral fat, were dissolved in dry chloroform and adsorbed on a column of silicic acid. Fig. 4 shows the separation of the lipids on elution with a continuous linearly increasing concentration of methanol in chloroform so adjusted that after 108 fractions of 25 ml. had been collected, the solvent in the mixing chamber was approx. 90% of methanol and 10% of chloroform. Each fraction of the eluate was analysed for phosphorus, total nitrogen and sugar; these are plotted as μg.atoms of P and N, and μmoles of sugar per fraction. The clotting activities were also determined.

The earlier fractions 17–49 which constitute the the first five peaks were poorly separated and after acid hydrolysis were found to contain over 90% of the total nitrogen as amino nitrogen; these fractions were further investigated after rechromatography. Fractions 50–108 contain choline as the base and were free from amino nitrogen. The results of rechromatography of these fractions is shown below.

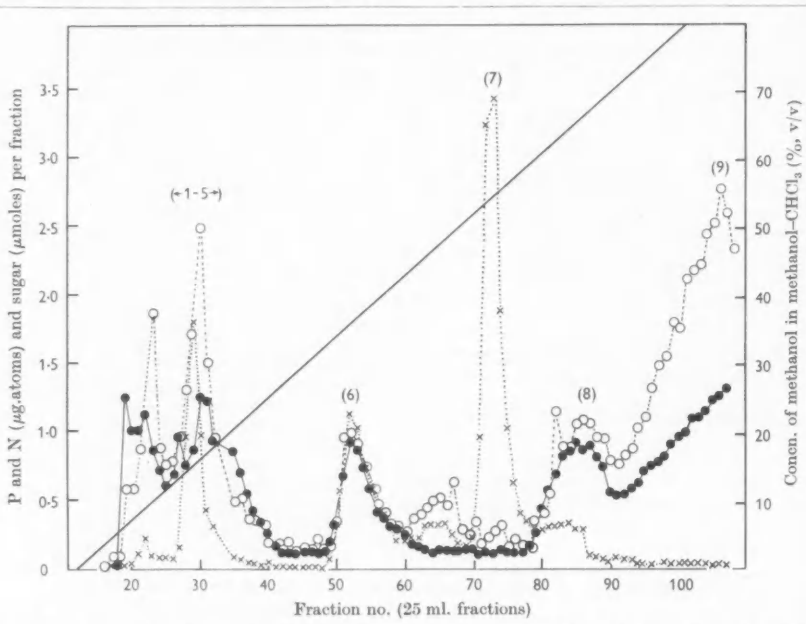

Fig. 4. Chromatography of butter phospholipids by a continuous linear gradient elution with methanol–$CHCl_3$ from a column of silicic acid. ●, Phosphorus; ○, nitrogen; ×, sugar; —, slope of the solvent gradient. Peak numbers are in parentheses.

Peak 6 (*fractions* 50–59, *Fig.* 4), *lecithin galactoside.* The intact lipid was detected on a silicic acid paper chromatogram with Rhodamine 6G and the phosphomolybdic acid reagent; its R_F was 0·44. The P:N:sugar ratios were approx. 1:1:1, and after hydrolysis the products identified were glycerophosphoric acid, choline and galactose. The lipid had no clotting activity.

Peak 7 (*fractions* 70–79, *Fig.* 4), *a sugar-containing phosphatidylcholine.* The lipid was detected by silicic acid paper chromatography with Rhodamine 6G and the phosphomolybdic acid reagent. The P:N ratio after reprecipitation (1 vol. of chloroform plus 10 vol. of acetone) was 1:1·2. After hydrolysis, galactose and glucose were detected, and the lipid had a clotting activity of 50 units/mg.

Peak 8 (*fractions* 82–89, *Fig.* 4), *lecithin.* The intact lipid on silicic acid paper chromatography was detected with Rhodamine 6G and phosphomolybdic acid; its R_F, 0·71, was identical with that of synthetic lecithin run alongside and in admixture. The P:N ratio, after reprecipitation as above, was 1:1·08 (theory requires 1:1) and acid-hydrolysis products were choline and glycerophosphate. The lipid had no clotting activity.

Peak 9 (*fractions* 100–108, *Fig.* 4), *sphingomyelin.* The intact lipid on silicic acid paper chromatography was detected with the Rhodamine 6G and phosphomolybdic acid reagents, R_F 0·61. It readily gave a reineckate and had a P:N ratio of 1:2·18 (theory 1:2). On acid hydrolysis followed by chromatography (as described for phosphate esters) the phosphate ester had R_F 0·8 (with reference to glycerophosphate R_F 1). Choline was liberated on prolonged acid hydrolysis, as described under hydrolysis for bases. The lipid had no clotting activity.

Rechromatography of peaks 1–5

Fractions 17–50 (Fig. 4), after removal of solvent, were dissolved in a small volume of chloroform, rechromatographed on a similar silicic acid column and eluted with a linear gradient of methanol in chloroform, so adjusted that after 60 fractions of 25 ml. a concentration of 30 % (v/v) of methanol in chloroform was reached in the mixing vessel. The separation of the early peaks (Fig. 5) was considerably improved, especially with respect to the sugar peak, and the nitrogen and sugar values of the fractions ran closely parallel with the phosphorus values. The peak fraction after reprecipitation as above gave the following results.

Peak 1 (*Fig.* 5), *a complex phosphatidic acid.* On silicic acid paper chromatography this gave no ninhydrin reaction and was detected with Rhodamine 6G and phosphomolybdate reagents, R_F 0·95 (considerable 'tailing'). The N content was negligible, and on hydrolysis no base was detectable and glycerophosphoric acid was obtained (tailing on chromatogram). The lipid had a weak clotting activity of 5 units/mg.

Peaks 2, 3 *and* 4 (*Fig.* 5), *phosphatidylserine and phosphatidylserine galactoside.* On silicic acid paper chromatography these peaks gave ninhydrin-positive spots which were also detectable with Rhodamine 6G (violet fluorescence under u.v. light) at R_F 0·73, 0·72, 0·72 respectively. The P:N ratio of each of the compounds was 1:0·87, 1:1·1 and 1:0·88 respectively, whereas peak 4 contained galactose (P:galactose ratio, 1:4·5). Hydrolysis of peaks 2 and 3 gave glycerophosphoric acid and serine whereas hydrolysis of peak 4 gave glycerophosphoric acid, serine and galactose. The clotting

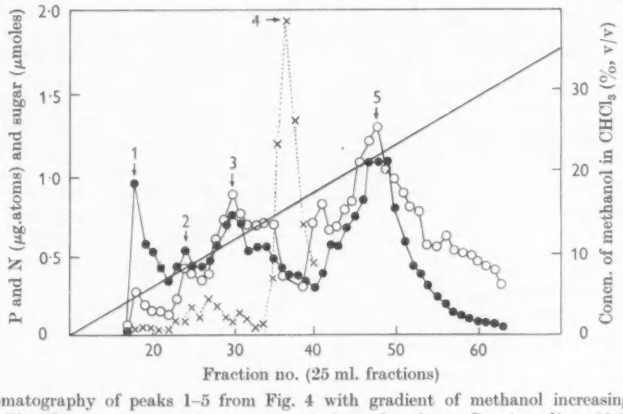

Fig. 5. Rechromatography of peaks 1–5 from Fig. 4 with gradient of methanol increasing to 34 % after 70 fractions. ●, Phosphorus; ○, nitrogen; ×, sugar; —, slope of gradient. Receiver diam. 83·5 mm. and donor 73 mm. Concentration of methanol in $CHCl_3$ in donor vessel, 57 % (v/v).

activities of peaks, 2, 3 and 4 were 85, 60 and 30 units/mg. respectively.

Phosphatidylserine appears as two distinct fractions (peaks 2 and 3) when run with a neutral solvent system whereas both compounds appear together when chromatographed on silicic acid paper with acid solvents.

Peak 5 (*Fig.* 5), *phosphatidylethanolamine, phosphatidylserine traces, compound* X. The lipid in this fraction appears to be the least homogeneous and on silicic acid paper chromatography gave three distinct components. (1) Phosphatidylethanolamine, R_F 0·76, which gave a positive ninhydrin reaction and with Rhodamine 6G a yellow fluorescent spot when viewed under u.v. light. Authentic L-α-dimyristoylphosphatidylethanolamine gave identical reactions and had R_F 0·74–76. (2) Phosphatidylserine, R_F 0·72, gave a ninhydrin-positive reaction and blue fluorescence with the Rhodamine 6G reagent when viewed under u.v. light. (3) Compound X, with R_F 0·54, gave a negative reaction with ninhydrin and phosphomolybdic acid reagent, but it appeared as a blue fluorescent spot with the Rhodamine 6G reagent under u.v. light. The mixed lipids showed a clotting activity of 500 units/mg. The mixture on hydrolysis showed the presence of ethanolamine and serine.

The mixture of phosphatidylethanolamine and phosphatidylserine was separated from compound X by cutting the paper. After elution of X, its clotting activity/mg. of P was 100 times as great as that of the mixed phosphatidylethanolamine and phosphatidylserine fraction.

Table 1. *Thromboplastic activity of butter phospholipids*

Peaks 1–5 refer to Fig. 5 and 6–9 to Fig. 4. Compound X [5 (*c*)] was approx. 100 times as active (per mg. of P) as the mixture of 5 (*a*) and 5 (*b*).

Peak no.	Phospholipid	Clotting activity (units/mg.)
1	A complex phosphatidic acid	5
2	Phosphatidylserine	85
3	Phosphatidylserine	60
4	Phosphatidylserine galactoside	30
5	Mixture: (*a*) Phosphatidylethanolamine, (*b*) Phosphatidylserine, (*c*) Unknown compound X	500
6	Phosphatidylcholine galactoside	0
7	A choline lipid containing glucose and galactose	50
8	Lecithin	0
9	Sphingomyelin	0
	Synthetic L-α-dimyristoylphosphatidylethanolamine	7

Summary of phospholipid fractions and their blood-clotting activities

A list of the phospholipids separated from butter is shown in Table 1, together with their clotting activities. It will be seen that the strongest clotting activity was in fraction 5 and, as shown above, the most active in this group is compound X. The synthetic L-α-dimyristoylphosphatidylethanolamine which was tested at the same time on the same plasma specimen exhibited only a weak activity.

DISCUSSION

Although much has been published on the analysis of butter and dairy fats (reviewed by Hilditch, 1949; Davis & Macdonald, 1953), little attention has been directed to the butter phospholipids which are described here. We have found that the whole of the strong thromboplastic activity of butter resides in a number of the phospholipid factions and these appear to be present as lipoproteins in the original butter. Ten phospholipids have been separated and six of these have thromboplastic activity. The most active, component X, which has been recognized only by silicic acid paper chromatography, remains unidentified; it was approximately 100 times as active as phosphatidylethanolamine and phosphatidylserine prepared from butter and far more active than synthetic phosphatidylethanolamine.

During the progress of this work Poole & Robinson (1956) and O'Brien (1956*a*) reported blood-thromboplastic activity from phospholipid fractions of egg and, when Lea *et al.* (1955) isolated phosphatidylethanolamine from the same source, the activity of egg yolk was attributed to its phosphatidylethanolamine content. In our experience egg yolk was only weakly active compared with butter, and as butter contains only traces of phosphatidylethanolamine its activity is unlikely to be due to this substance. It appears from the evidence presented here that phospholipids other than phosphatidylethanolamine are strong potentiators of blood coagulation. The difficulty of assessing the activity of thromboplastic agents has been overcome by the use of an arbitrary system of blood-clotting units where butter has been chosen as a standard of comparison, not only because it was the most active fat, but also because a large number of samples of butter tested had approximately the same thromboplastic activity.

Silicic acid chromatography has long been recognized as a powerful tool in the separation of phospholipids and although various techniques have been reported with this absorbent, it appears that the choice of the method of elution is dependent on the composition of the lipids requiring

separation (Lea *et al.* 1955); Hanahan, Dittmer & Warashina, 1957; Marinetti, Scaramuzzino & Stotz, 1957). With the Lea *et al.* method the butter phospholipids were eluted as a single peak, and further experience indicated the need for a gradient elution system.

When a modified exponential system of elution, as used by Alm, Williams & Tiselius (1952) for the separation of fatty acid mixtures, was used with the methanol–chloroform solvent system, the concentration of the polar solvent increased too rapidly in the early stages of chromatography. The linear gradient chromatography eventually used in this work is a modification of the systems of Kellie & Wade (1957) and Edwards (1958). The large differences in the densities of methanol and chloroform necessitated the use of donor and receiver vessels of different cross-sectional areas. With such a gradient elution system, highly reproducible chromatographic separation was obtained and preliminary experiments on the separation of phospholipids from egg yolk and blood plasma suggest that the method may be of general use in the fractionation of phospholipids.

The fatty acid composition of the phospholipids has not been investigated and the nature of the sugar linkage in the glycosides is uncertain, although it has been established that the sugar is not present in a free state and is probably linked through the amino nitrogen in the 'kephalin' group of lipids. Phosphatidylserine appears as two distinct peaks (peaks 2 and 3, Fig. 5) and it is unlikely that separation within this group is obtained through a difference in fatty acid compositions, for on silicic acid paper chromatography in acid media the two components run together. This observation is in agreement with that of Marinetti, Erbland & Stotz (1958), who found two ionic forms of phosphatidylserine in brain phospholipids.

The physiological importance of the butter phospholipids described is uncertain since there is evidence that such compounds are probably modified during intestinal absorption (Ahrens & Borstrom, 1956; O'Brien, 1957; Maclagan, Billimoria & Curtis, 1958). Nevertheless, the possible influence of dietary phospholipids on blood phospholipids is of some interest and is under further investigation.

SUMMARY

1. The addition of butter to plasma shortened the Stypven clotting time of human plasma by over 75% and butter was more active than any other fat tested.

2. A preliminary separation of butter lipids indicated that the thromboplastic activity was confined to the phospholipids originally bound to the proteins of butter.

3. Butter phospholipids were separated by chromatography on silicic acid columns by stepwise and continuous gradient elution techniques, with methanol–chloroform mixtures. A simple method of obtaining an approximately linear gradient is described which may be of general use in the separation of phospholipid mixtures.

4. Ten major phospholipids have been detected in butter and most of these have been identified by paper chromatography and chemical analysis of the intact lipids and their hydrolysis products.

5. A comparison of the Stypven clotting activities of the butter-phospholipid fractions with that of synthetic L-α-dimyristoylphosphatidylethanolamine has shown that a number of these fractions are far more active than the synthetic compound. The most active butter phospholipid (compound X), has not yet been identified.

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On the Mechanism of α-Oxoglutarate Oxidation in *Escherichia coli*

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The enzymic formation of succinyl-coenzyme A from α-oxoglutarate is known to require diphosphopyridine nucleotide (DPN^+), thiamine pyrophosphate (TPP), lipoic acid (lip-$S_{ox.}$ or lip-$S_{red.}$) and coenzyme A (CoA). It has been postulated (Gunsalus, 1953; Kaufman, 1955; Sanadi, Langley & White, 1959) that the sequence of reactions involved in the overall reaction is:

(1) α-oxoglutarate + TPP ⇌ [succinic semialdeyde]-TPP + CO_2;

(2) [succinic semialdehyde]-TPP + lip-$S_{ox.}$ ⇌ succinyl-S-lip + TPP;

(3) succinyl-S-lip + CoA-SH ⇌ succinyl-S-CoA + lip-$S_{red.}$;

(4) lip-$S_{red.}$ + DPN^+ ⇌ lip-$S_{ox.}$ + reduced DPN + H^+.

Sum:

(5) α-oxoglutarate + DPN^+ + CoA-SH ⇌ succinyl-S-CoA + reduced DPN + H^+ + CO_2.

Attempts to isolate the enzymes catalysing these individual steps have been largely unsuccessful, although lipoic dehydrogenase (reaction 4) has been separated and shown to be a component of the α-oxoglutarate-dehydrogenase system of *Escherichia coli* (Hager & Gunsalus, 1953). More recently the mammalian α-oxoglutarate-dehydrogenase system has also been resolved with respect to lipoic dehydrogenase (Massey, 1960) and it has been shown to be a flavoprotein (Massey, 1958; Searls & Sanadi, 1959) identical with Straub's (1939) diaphorase (Massey, 1960).

It is the purpose of this paper to characterize the biochemical lesion in a succinate-requiring mutant of *E. coli*. The experiments reported indicate that the mutant lacks the enzyme catalysing reaction 1, α-oxoglutarate carboxylase, or reactions 1 and 2. It therefore provides a source for the α-oxoglutarate-dehydrogenase complex which is deficient in this enzyme or these enzymes.

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METHODS AND MATERIALS

Maintenance of stock culture. *E. coli* W (wild type) and the succinate-requiring mutant (*E. coli* 309–1 R) were gifts from Professor B. D. Davis (Harvard University Medical School, Boston, Mass., U.S.A.). The organisms were maintained in stock culture on agar slopes containing (per 100 ml. of medium) 1 g. of tryptone, 1 g. of Difco yeast extract, 0·5 g. of dipotassium hydrogen phosphate, 0·3 g. of glucose, 0·2 g. of sodium succinate and 2 g. of agar agar (Hopkin and Williams Ltd., Chadwell Heath, Essex).

Preparation of inoculum and growth of cells. Both wild-type and mutant cells were grown in an identical manner. The inoculum was prepared by transferring cells from the stock-culture agar slopes to 50 ml. of liquid medium containing the same ingredients (minus agar) as the agar slopes. These cultures were incubated aerobically with shaking overnight at 30° and served as inocula for growth carboys (vol. 10 l.). The growth medium contained (per 100 ml.) 0·1 g. of Difco yeast extract, 0·1 g. of tryptone, 0·7 g. of dipotassium hydrogen phosphate, 0·3 g. of potassium dihydrogen phosphate, 0·1 g. of ammonium sulphate, 0·01 g. of $MgSO_4,7H_2O$, 0·5 g. of glucose and 0·05 g. of sodium succinate. The 10 l. cultures were grown overnight at 30° with forced aeration aided by sintered-glass spargers. The cells were harvested by centrifuging in a Sharples centrifuge and the cell paste was stored at −20°. The yield of wild-type cells was 65 g. of cell paste/10 l. of culture. The yield of mutant cells was 40 g. of cell paste/10 l. of culture.

Preparation of cell-free extracts. Cell-free extracts of both wild-type and mutant cells were prepared by suspending 2 g. of cell paste in 10 ml. of 0·02 M-2-amino-2-hydroxymethylpropane-1:3-diol(tris) buffer, pH 7·0, and subjecting the resultant slurry to the output of a 600 W Mullard magnetostrictor oscillator (operating at 3·5 A, 25 kcyc./sec.) for 3 min. Unbroken cells and cell debris were removed by centrifuging at 20 000 *g* for 15 min. at 4° in a PR-2 International refrigerated centrifuge. The pellet obtained from the centrifuging was discarded. The supernatant fraction was dialysed overnight against 100 vol. of 0·02 M-tris buffer, pH 7·0, and subsequently stored at −20°.

Substrates and cofactors. α-Oxoglutarate was a gift from Professor Sir Hans Krebs, F.R.S.; DPN and CoA were obtained from the Sigma Chemical Co., St Louis, Mo., U.S.A.; TPP and oxidized lipoic acid were purchased from the California Research Foundation; the last-named compound was reduced according to the method of Gunsalus, Barton & Gruber (1956). Sodium [^{14}C]bicarbonate was prepared from $Ba^{14}CO_3$, obtained from The Radiochemical Centre, Amersham, Bucks., as previously described (Kornberg, Davies & Wood, 1952).

RESULTS

Oxidation of α-oxoglutarate with diphosphopyridine nucleotide as electron acceptor and coenzyme A as acyl acceptor. Crude extracts of wild-type *E. coli* catalyse the overall oxidation of α-oxoglutarate to form reduced diphosphopyridine nucleotide (DPNH) and succinyl-CoA (reaction 5). Fig. 1, curve 1, shows the formation of DPNH from α-oxoglutarate, in the presence of substrate amounts of CoA, catalysed by the crude extract prepared from wild-type cells. The extract prepared from the succinate-requiring mutant failed to catalyse this reaction (Fig. 1, curve 3), as would be expected, since growth experiments (Davis, Kornberg, Nagler, Miller & Mingioli, 1959) predict that the mutant lacks one of the enzymes involved in the oxidation of α-oxoglutarate to succinate. A mixture of the mutant extract plus the wild-type extract formed DPNH at the same rate as did the wild-type extract alone (Fig. 1, curve 2). This finding indicates that the mutant extract lacks an enzyme of the α-oxoglutarate-dehydrogenase system, and that its failure to catalyse the overall

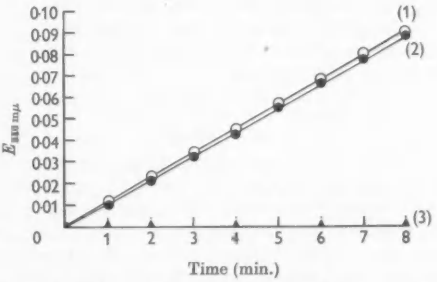

Fig. 1. Oxidation of α-oxoglutarate with DPN as electron acceptor and CoA as acyl acceptor. The complete system contained, in 3 ml.: 300 μmoles of potassium phosphate buffer, pH 7·0, 200 μmoles of magnesium chloride, 0·5 μmole of thiamin pyrophosphate, 100 μmoles of cysteine, 1 μmole of coenzyme A, 0·3 μmole of diphosphopyridine nucleotide, 100 μmoles of α-oxoglutarate, and cell-free extracts. 1 (○), Wild-type extract (6 mg. of protein); 2 (●), wild-type extract plus mutant extract (6 mg. of protein of each); 3 (▲), mutant extract (6 mg. of protein). Control cuvettes contained the above mixtures with DPN omitted. All reactions were run anaerobically at room temperature. The oxidation of DPN was measured in a Beckman spectrophotometer as the increase in extinction at 340 mμ.

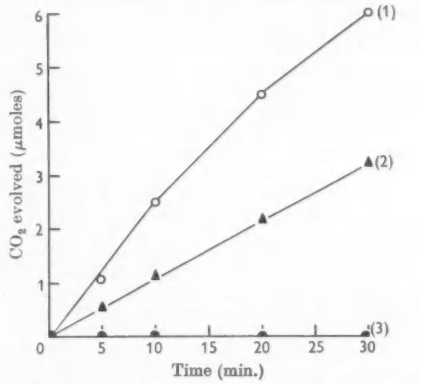

Fig. 2. Oxidation of α-oxoglutarate with ferricyanide as electron acceptor. The complete system contained (in a total volume of 1 ml.): 100 μmoles of potassium phosphate buffer, pH 6·0, 20 μmoles of sodium α-oxoglutarate, 0·5 μmole of TPP, 25 μmoles of potassium ferricyanide plus wild-type extract (12 mg. of protein: curve 1), wild-type extract (6 mg. of protein: curve 2) or mutant extract (12 mg. of protein: curve 3). The reaction mixtures were incubated at 30° for 30 min. under N_2 and the rate of CO_2 evolution was measured in Warburg manometers.

reaction is not due to an inhibitor of the enzyme complex in the mutant extract.

Oxidation of α-oxoglutarate with ferricyanide as electron acceptor. A comparison of the mutant and wild-type extracts for the ability to effect the oxidative decarboxylation of α-oxoglutarate with ferricyanide as electron acceptor is shown in Fig. 2. It is evident that, whereas the wild-type extract catalysed the oxidative decarboxylation of α-oxoglutarate at rates proportional to the amounts of extract added, the extract prepared from the mutant did not catalyse this reaction. Since TPP is the only cofactor required for its occurrence, it has been postulated (Gunsalus, 1953; Schweet, Fuld, Cheslock & Paul, 1953) that this reaction is a measure of α-oxoglutarate-carboxylase activity (reaction 1). The finding that the mutant did not catalyse the ferricyanide-linked reaction therefore offers circumstantial evidence about the metabolic lesion in the mutant.

Comparison of the wild-type and mutant extracts for their ability to catalyse $^{14}CO_2$ exchange with α-oxoglutarate. The exchange of $^{14}CO_2$ with α-oxoglutarate provides a more reliable assay for α-oxoglutarate-carboxylase activity. It has been previously established that TPP is the only cofactor requirement for this exchange reaction (Goldberg & Sanadi, 1952). Furthermore, it has been shown that $^{14}CO_2$ exchange with pyruvate is a measure of the carboxylase activity of the pyruvate-dehydrogenase complex (A. D. Gounaris & L. P. Hager, unpublished experiments). The ability of extracts of the wild type and the mutant organisms to catalyse the exchange of $^{14}CO_2$ with α-oxoglutarate was tested as described below.

The complete system contained 200 μmoles of magnesium chloride, 0·5 μmole of TPP, 10 μmoles of sodium α-oxoglutarate, 2 μmoles of $NaH^{14}CO_3$ (containing 2×10^6 counts/min.) and wild-type or mutant extract (20 mg. of protein of each) in a total volume of 1·8 ml. The reaction mixtures were incubated at 30° for 1 hr. under nitrogen. After incubation, the reactions were stopped by the addition of 0·6 ml. of 6N-HCl, and denatured protein was removed by centrifuging. A saturated solution of 2:4-dinitrophenylhydrazine in 2N-HCl (1 ml.) was added and the solution was allowed to stand at room temperature for 2 hr. The 2:4-dinitrophenylhydrazone of α-oxoglutarate together with unreacted 2:4-dinitrophenylhydrazine were extracted from the acid solution by four extractions of 2 ml. each of ethyl acetate. The ethyl acetate extract was concentrated to a small volume, approx. 0·5 ml., under nitrogen at 60°. The concentrated solution was applied as a streak to Whatman no. 3 filter paper and chromatographed in the solvent of El Hawary & Thompson (1953). The α-oxoglutarate 2:4-dinitrophenylhydrazone was eluted from the paper with 5% sodium bicarbonate. The sodium bicarbonate solution was acidified with HCl and the hydrazone extracted into ethyl acetate as described above; the ethyl acetate solution was then concentrated and plated on aluminium planchets for radioassay.

The 2:4-dinitrophenylhydrazone of α-oxoglutarate isolated after incubation with the wild-type extract gave 200 counts/min., but that isolated after incubation with the mutant extract was devoid of radioactivity. Hence, whereas extracts of the wild type catalyse $^{14}CO_2$ exchange with α-oxoglutarate, extracts of the mutant failed to catalyse this exchange reaction.

Other enzymes associated with the α-oxoglutarate-dehydrogenase complex. Both the wild-type extract and the mutant extract contained lipoic dehydrogenase (Table 1). The lipoic-dehydrogenase content of the extracts was estimated by following the disappearance of reduced lipoic acid in the presence of catalytic amounts of DPN plus pyruvate and lactic dehydrogenase (Hager & Gunsalus, 1953).

In addition, both the wild-type and mutant extracts contained succinic thiokinase, as assayed by the hydroxamic acid-trapping method (Kaufman, 1955). The mutant extract contained approximately 75% of the succinic-thiokinase activity of the wild-type extract (Table 1). Advantage was taken of the fact that both extracts contained succinic thiokinase in developing an assay for lipoic trans-succinylase. Succinic thiokinase was employed to generate catalytic amounts of succinyl-CoA according to equation 6:

(6) ATP + succinate + CoA-SH → adenosine monophosphate + pyrophosphate + succinyl-S-CoA.

The transfer of the succinyl moiety from CoA to reduced lipoic acid, to yield succinyl lipoic acid (reaction 7),

(7) Succinyl-S-CoA + lip-$S_{red.}$ → CoA-SH + succinyl-S-lip,

was then detected by the lipoic acid-dependent formation of succinyl hydroxamic acid. In this case, hydroxylamine was added at the end of the

Table 1. *Lipoic-dehydrogenase and succinic-thiokinase activity of wild-type and mutant extracts*

Lipoic-dehydrogenase activity was assayed by the system developed by Hager & Gunsalus (1953); specific activity is defined as μmole of reduced lipoic acid oxidized/hr./mg. of protein. Succinic-thiokinase activity was assayed by the hydroxamic acid method (Kaufman, 1955); specific activity refers to μmole of succinic hydroxamate formed/hr./mg. of protein.

Extract	Specific activity: Lipoic dehydrogenase	Specific activity: Succinic thiokinase
Wild type	0·80	0·19
Mutant	0·88	0·14

incubation period. Some succinyl hydroxamate formation took place in the absence of lipoic acid since catalytic amounts of succinyl-CoA were generated by succinic thiokinase. In addition, both the wild-type and mutant extracts gave a colour in the ferric chloride test for succinyl hydroxamate. These two factors combine to yield the apparent 'succinyl hydroxamate' at zero time shown in Fig. 3. However, the intensity of this colour did not increase with time in the absence of lipoic acid: lipoic trans-succinylase action is shown as the formation as a function of time of succinyl hydroxamate, which depended on the presence of succinate and lipoic acid (Fig. 3). The two extracts have essentially equal, but low, catalytic activities in this assay system.

Succinate formation from malate under anaerobic conditions. Growth studies (Davis *et al.* 1959) have shown that the succinate-requiring mutant will grow anaerobically and utilize glucose in the absence of succinate. This finding implies that the mutant is capable of forming succinate under anaerobic conditions, presumably by the reduction of oxaloacetate, malate and fumarate. Undialysed cell-free extracts of the mutant were tested for their ability to convert [^{14}C]malate (Dixon, Kornberg & Lund, 1960) into succinate under anaerobic conditions. The crude, undialysed extract, incubated for 1 hr. under nitrogen with tris buffer and magnesium chloride but without added electron donors, catalysed the conversion of approximately 80% of added [^{14}C]malate into succinate: cell-free extracts of the mutant therefore reflect the ability of the intact cell to effect the anaerobic synthesis of succinate from malate.

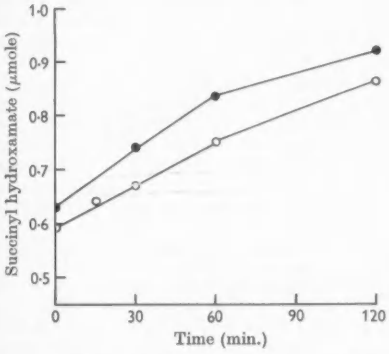

Fig. 3. Formation of succinyl hydroxamate dependent upon the presence of ATP, CoA, succinate and reduced lipoic acid. The complete system contained (in 1 ml.): 100 μmoles of potassium phosphate, pH 7·5, 100 μmoles of ATP, 0·2 μmole of CoA, 20 μmoles of sodium borohydride, 10 μmoles of reduced DL-lipoic acid and 8 mg. of wild-type extract (○) or 8 mg. of mutant extract (●). Reaction mixtures were incubated at 30°. At the time intervals indicated 800 μmoles of neutralized hydroxylamine were added and the mixture was allowed to stand at room temperature for 5 min. Succinyl hydroxamate colour was then developed by the addition of $FeCl_3$ reagent (Lipmann & Tuttle, 1945). The reading at zero time indicates the colour formed in an incubation mixture containing all the components of the complete system but with either succinate or reduced lipoic acid omitted: the intensity of this colour did not increase with time.

DISCUSSION

Experimental verification of the proposed four-step reaction for the oxidation of pyruvate and α-oxoglutarate linked to DPN, CoA and lipoic acid has been hampered by the lack of success in separating the individual components. Both the pyruvate- and α-oxoglutarate-dehydrogenase systems fractionate as complexes from both bacterial and mammalian sources. At this time it is not known whether the complex formation primarily involves covalent bonding or merely electrostatic interaction, although with the lipoic-dehydrogenase component of the α-oxoglutarate complex it appears that electrostatic interaction is involved (Massey, 1960).

The isolation of mutants of *E. coli* requiring acetate or succinate offers a unique approach to the resolution and study of the pyruvate- and α-oxoglutarate-dehydrogenase complexes. Genetic considerations render it probable that these mutant organisms specifically lack only one of the sequential enzymes; they therefore offer a source of the enzyme complex resolved with respect to one of its components.

The experiments reported in this paper indicate that the succinate-requiring mutant (309-1R) of *E. coli* specifically lacks the 'α-oxoglutarate carboxylase' portion of the α-oxoglutarate-dehydrogenase complex. Extracts of the mutant, in contrast with those of the wild-type organism, failed to catalyse the exchange of $^{14}CO_2$ into α-oxoglutarate and the ferricyanide-linked oxidation of α-oxoglutarate as well as the overall formation of DPNH and succinyl-CoA from α-oxoglutarate. Since both the wild-type and the mutant extracts contain lipoic dehydrogenase, lipoic trans-succinylase and succinic thiokinase, it is probable that the primary decarboxylative step in the oxidation of α-oxoglutarate is ascribable to a discrete enzyme in the complex of enzymes not normally resolvable.

SUMMARY

1. Cell-free extracts of the succinate-requiring mutant of *E. coli*, 309-1R, and of its parent wild-type *E. coli* W, were tested for their ability to catalyse reactions involved in the overall oxidation of α-oxoglutarate to succinate.

2. Whereas extracts of the wild type catalysed the reduction of diphosphopyridine nucleotide in the presence of α-oxoglutarate, thiamin pyrophosphate and coenzyme A, extracts of the mutant did not. Addition of mutant extract to that of the wild type did not affect the latter's ability to oxidize α-oxoglutarate; this shows that the inability of the mutant extract to effect this reaction was due not to the presence of an enzymic inhibitor, but to the lack of an enzymic component of the α-oxoglutarate-dehydrogenase system.

3. Extracts of the wild-type organism catalysed the anaerobic evolution of carbon dioxide from α-oxoglutarate in the presence of thiamin pyrophosphate and ferricyanide as electron acceptor. The rate of gas evolution was proportional to the amounts of extract added. Extracts of the mutant failed to effect this reaction.

4. Extracts of the wild-type organism in the presence of thiamin pyrophosphate catalysed the incorporation of isotope from [^{14}C]bicarbonate into α-oxoglutarate, by isotopic exchange. Extracts of the mutant did not catalyse this reaction.

5. Extracts of both types of organism contained succinic thiokinase, lipoic dehydrogenase and lipoic trans-succinylase: a new assay procedure for the last-named enzyme is reported.

6. These results show that the mutant lacked the enzymes catalysing the first step or first two steps in the overall oxidation of α-oxoglutarate. They also indicate that the formation of the succinic semialdehyde–thiamin pyrophosphate complex and carbon dioxide from α-oxoglutarate is effected by a discrete enzymic component of the α-oxoglutarate-dehydrogenase system.

7. Undialysed cell-free extracts of the mutant, in the absence of added electron donors, catalysed the anaerobic reduction of [^{14}C]malate to [^{14}C]-succinate: this reflects the ability of the mutant to grow anaerobically even in the absence of succinate.

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The Measurement of Aconitase Activity in the Leaves of Various Normal and Variegated Plants

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Aconitase, an enzyme widely distributed in living organisms, was first found in higher plants (kidney beans, cucumber seeds) by Martius (1939), and was later shown to occur in leaves of cabbage (Jacobsohn & Soares, 1940) and of rhubarb (Morrison & Still, 1947).

Studies by Dickman & Cloutier (1951) and by Morrison (1954) have shown that during its purification from animal tissues the enzyme loses activity, which can be restored by the addition of ferrous iron together with cysteine, thioglycollate, ascorbate or glutathione. It has therefore been

assumed that *in vivo* the enzyme is associated with an iron compound which is lost or inactivated during the process of purification. Although no observations on this point have been made with plant aconitase, one of us has suggested (DeKock, 1954–55) that aconitase in plants might also be associated with iron, and hence that iron-deficient plants might contain less aconitase than normal ones.

We have developed a procedure, based on that of Racker (1950), for measuring aconitase activity in leaves. This paper describes its application to various species of plants, including some with variegated foliage. Its application to iron-deficient plants has been reported to the Biochemical Society (Bacon, DeKock & Palmer, 1959).

MATERIALS AND METHODS

Unless otherwise stated plants were grown in the gardens or greenhouses of this Institute. From September to April the temperature of the greenhouses was kept between 5° and 20°, and extra illumination was provided by groups of fluorescent discharge tubes from 5 a.m. until 9 p.m. each day. Leaves were picked shortly before use, and were conveyed to the laboratory in a dry beaker surrounded by crushed ice in a vacuum flask. The weighed samples were sliced with plastic scissors before being disintegrated in a suitable extraction medium (see below).

Substrates. Solutions of (±)-*iso*citrate were prepared by the hydrolysis of (±)-*iso*citric acid lactone (L. Light and Co. Ltd.) with 1·5 N-NaOH at 100° for 30 min., and subsequent neutralization with 2 N-HCl to pH 8·5. Citrate, malate and *cis*-aconitate solutions were made by dissolving citric acid, malic acid and *cis*-aconitic acid anhydride (prepared by the method of Malachowski & Maslowski, 1928) respectively in water and adjusting the pH to 8·5 with N-NaOH. Oxaloacetic acid was a commercial preparation (L. Light and Co. Ltd.) or was prepared from sodium diethyloxaloacetate (Eastman Kodak Ltd.) as described by Heidelberger (1953).

Estimations. Citrate was estimated by the method of Taylor (1953), and ascorbic acid by titration with 2:6-dichlorophenolindophenol (Roe, 1954) at a dye concentration of 0·1 mg./ml.

RESULTS

Measurement of aconitase activity

First attempts to measure aconitase activity directly in the spectrophotometer by the addition of leaf extract to the buffered citrate or *iso*citrate solutions described by Racker (1950) were not successful. Incubations were therefore made with larger proportions of leaf extract, and small samples were deproteinized before measurement of the absorption at 240 mμ. In this way it was found that for optimum activity the leaf enzyme required a higher pH and substrate concentrations about ten times as great. On the basis of these results the following procedure was then developed: 0·5 g. of leaf was disintegrated in an all-glass Potter–Elvehjem homogenizer with 2·5 ml. of extraction medium previously cooled in ice. The extraction medium contained 0·5 M-sucrose, 0·2 M-2-amino-2-hydroxymethylpropane-1:3-diol(tris)–HCl buffer (pH 8·5) and 5 mM-ethylenediaminetetra-acetic acid (EDTA). The suspension was passed through muslin into a cooled tube and centrifuged at about 1300 *g* for 5 min., during which time the temperature rose to about 15°. The supernatant fluid was decanted into a cooled tube and kept on ice. Two 1 cm. cells of the spectrophotometer (Unicam SP. 500) were set up, one containing sodium (±)-*iso*citrate, and both a sucrose solution buffered with tris–HCl at pH 8·0. After addition of the leaf extract (0·025–0·10 ml.) the final concentrations were: 0·05 M-(±)-*iso*citrate, 0·5 M-sucrose and 0·1 M-tris, in a total volume of 3 ml. The *iso*citrate solution itself contributed to the mixture an extinction of 0·4–0·8 at 240 mμ depending upon the batch of lactone used. Addition of leaf extract to both cells increased the extinction considerably, so that with some extracts more than 0·05 ml. made the instrument so insensitive that readings were unreliable. Usually, however, it was possible to measure an increase of extinction (240 mμ) of between 0·05 and 0·50 unit during a 30 min. period after the addition of the extract: in some extreme cases the change was as great as 1·0 unit with 0·05 ml. of extract (*Sambucus*). The temperature was controlled at 25° in the constant-temperature cell holder supplied with the Unicam SP. 500. The increase was almost always linear with time, and from it the μmoles of aconitate formed were calculated from an extinction value given by Racker (1950) of 0·71 for 0·2 mM-*cis*-aconitate. Aconitase activities are expressed as μmoles of *cis*-aconitate formed/g. fresh wt. of leaf/hr. (Table 2).

On the assumption that all the measured extinction increment was the result of aconitase activity, the following factors were examined for their influence on the extractability of the enzyme. (The species on which a particular test was performed is given in parentheses.) Sampling error, which is great when only 0·5 g. of leaf is taken, was largely overcome by using opposite leaflets, or half-leaflets from the same leaf (tomato, broad bean) or opposite sides of simple leaves (mustard). Extracts prepared in this way showed good agreement of activities, often better than ±5%.

Method of disintegration. The use of a large (21 cm. diam.) mortar and pestle, cooled overnight in the refrigerator, with or without sand, gave extracts of similar activity to those prepared in the homogenizer. Extension of treatment beyond the time needed to disintegrate all large particles tended, if anything, to decrease the activity.

Extraction medium. Sucrose was originally included with the intention of preserving the mitochondria, but later experiments showed that practically all the aconitase activity was present in the supernatant after centrifuging at 25 000–40 000 *g* for 15 min.

Tris buffer was preferred to phosphate because in preliminary experiments the latter seemed to depress the activity (cf. Pierpoint, 1959), but later tests with improved technique showed no appreciable difference. Sodium EDTA has a strong absorption at 240 mμ and contributes an extinction of about 0·02 when 0·05 ml. of extract is added to a final volume of 3·0 ml. of buffered substrate. It was retained in the extraction medium as a safeguard against heavy-metal contamination. It delayed the darkening of some leaf extracts (e.g. broad bean) but did not prevent a slow increase in absorption at 240 mμ. Its omission did not seem to affect the aconitase activity (cabbage, *Abutilon*). Addition of citrate to the medium (Pierpoint, 1959) increased the activity by 5–10 % (*Abutilon*).

pH *and molarity of buffer.* The pH of the final extract was usually somewhat lower (at most 0·5 unit) than that of the extraction medium, in which the buffer concentration was 0·2 M. Experiments with cabbage and *Abutilon*, and more extensive experiments with tomato leaves, showed that much the same activity was measured after extraction with media of pH between 8·0 and 9·0, but that distinctly lower values (50–75 %) were found with media of pH 7·0–7·5.

Centrifuging at 1300 *g.* This removes cell debris, chloroplast material, starch granules, etc. When it was omitted (*Abutilon*) the extract appeared darker and more opaque, but did not absorb much more at 240 mμ: the aconitase activity was somewhat higher (about 10 %).

Storage of the low-speed supernatant. Even at 0°, extracts of some leaves lost activity fairly rapidly. Thus a tomato-leaf extract lost one-quarter of its activity in 7 hr. at 0°, one-half in 6 hr. at 25° and more than two-thirds in 4·5 hr. at 40°. Extracts from cabbage and mustard leaves were much more stable. The activity lost in this way could not be restored by treatment with ferrous ammonium sulphate and cysteine at 0° (Morrison, 1954).

Composition of the test medium. The activity had a broad optimum between pH 7·5 and 8·5 (cabbage, broad bean). The omission of sucrose or replacement of the tris–HCl by phosphate did not affect the activity. Activity was not a maximum at the concentration of *iso*citrate used; no precise measurements of K_m were made, but it was estimated to lie between 0·5 and $1·0 \times 10^{-2}$ M-(±)-*iso*-citrate (broad bean, parsley, *Abutilon*).

Intracellular location of enzyme

It was thought that if the aconitase were associated with a particulate fraction it might be separated from the crude extracts by high-speed centrifuging, but tests on various species showed that this was not feasible. Centrifuging at 40 000 *g* in a refrigerated centrifuge for periods up to 1 hr. yielded a pellet which usually contained only 5–15 % of the total activity (Table 1). The activity in the pellet was clearly more than could be accounted for by the supernatant fluid it contained, but it also seemed to be less stable, and the point was not investigated further. Simultaneous estimation of fumarase in the cabbage extract showed that a rather greater proportion of the total (10–15 %) was associated with the pellet.

Evidence for the presence of aconitase

The increase in extinction at 240 mμ in the presence of *iso*citrate is only presumptive evidence for the formation of *cis*-aconitate. We therefore tested other aspects of the reactions catalysed by aconitase: the formation of *cis*-aconitate from citrate, the disappearance of added *cis*-aconitate and the formation of citrate from either *iso*citrate

Table 1. *Distribution of aconitase activity after high-speed centrifuging of leaf extracts*

Crude extracts were centrifuged for 15 min. at 25 000–40 000 *g* in a refrigerated centrifuge. Each sediment was resuspended in the original volume of extraction medium, and 0·05 ml. samples were taken for each assay.

Species	pH of extraction medium	Aconitase activity (μmoles/g. fresh wt./hr.) Whole extract	Supernatant (*a*)	Sediment (*b*)	Percentage in sediment [100 *b*/(*a* + *b*)]
Vicia faba	8·5	43	40	6	13
	8·5	30	23	5	19
	7·5	45	38	5	12
	7·5	55	43	3	7
Brassica oleracea	8·5	—	21	1	5
	8·5	—	21	1	5
	8·5	—	24	1	4
Sinapis alba	8·5	—	24	5	17

or *cis*-aconitate. With citrate as substrate there was also an increase in extinction at 240 mμ, but this was always less than that observed with the standard concentration of *iso*citrate, even when high concentrations of citrate were used. A series of comparisons was made with extracts of broad-bean leaves having different activities in the standard test (figures in parentheses), at the concentrations of the two substrates [10 mM-(±)-*iso*citrate; 30 mM-citrate] selected by Racker (1950), and the following ratios of activities were found: 1·6 (55), 1·5 (41), 2·0 (74) and 1·7 (82). With 0·25 mM-*cis*-aconitate as substrate there was a fall in extinction comparable with the increase with 10 mM-(±)-*iso*citrate; for three broad-bean extracts with a wide range of activity the ratios of the activities towards *cis*-aconitate and *iso*citrate were 0·68, 1·0 and 1·1. Estimations of citrate showed that it was formed under the assay conditions at a rate comparable with the rate of conversion of *iso*citrate and *cis*-aconitate. Dr R. I. Morrison kindly examined the products of an incubation of leaf extract with *iso*citrate by partition chromatography on silica gel (DeKock & Morrison, 1958), and showed that both citrate and *cis*-aconitate were formed. The agent or agents responsible for all these changes were destroyed by heating the extract, or even, with some extracts (e.g. tomato), by leaving them overnight at room temperature.

Effect of inhibitors of aconitase. Potassium cyanide at concentrations from 0·4 to 50 mM had an inhibitory effect on extracts of various species (broad bean, cabbage, tomato, mustard, *Heracleum*, *Hosta*, *Sambucus*). In these experiments neutral potassium cyanide solutions were added to the extracts at 0° to give the concentration stated, and 0·05 ml. samples were withdrawn at varying intervals (up to 2 hr.) for tests in the spectrophotometer. In very few cases was the activity completely inhibited, and in the majority the degree of inhibition varied, even from extract to extract of the same species, as might be expected with crude extracts. Extracts treated with 1–10 mM-cyanide for 30 min. at 0° lost 50–75 % of their activity. The increase in extinction during the enzyme assays was always linear, suggesting that no reversal of inhibition took place when the extract was diluted. The inhibitory effects of αα′-dipyridyl and *o*-phenanthroline were slow to develop (cf. Dickman & Cloutier, 1951). In these cases inhibition was indicated by a quicker loss of activity in the presence of the inhibitor than in its absence when the extract was kept at 0° for periods of 2–6 hr. (*Abutilon*).

Aconitase activities in the leaves of various plants

In view of the above findings it seemed probable that the procedure adopted would permit a comparison of the relative aconitase activities of leaves. The activities measured for fully expanded leaves of plants grown in soil are given in Table 2. In view of the effects of leaf age and position, one should not attach too much importance to the mean values given.

Particularly high values were found for two variegated plants, *Sambucus nigra variegata* (see Table 4) and *Abutilon striatum thompsonii*. The succulents tested all gave low or zero values by the standard procedure; the low absorption of these extracts made it possible sometimes to test as much as 0·25 ml. Previous vacuum-infiltration

Table 2. *Aconitase activities of extracts of green leaves of various species*

The mature leaves of plants grown in soil in the open or in the greenhouse were tested as described in the text. The plants are divided into three groups: crops of economic importance, garden plants and succulents.

Species	Common name	No. of extracts tested	Aconitase activity (μmoles/g. fresh wt./hr.) Range	Average
Allium cepa	Onion	1	—	55
Avena sativa	Oats	1	—	35
Brassica oleracea	Cabbage	5	32–60	47
Lycopersicum esculentum	Tomato	28	21–86	46
Sinapis alba	Mustard	8	15–34	27
Vicia faba	Broad bean	16	15–55	33
Abutilon striatum	—	18	49–164	92
Corydalis lutea	Yellow fumitory	2	80–118	99
Digitalis purpurea	Foxglove	1	—	49
Heracleum giganteum	—	3	71–84	80
Petroselinum hortense	Parsley	3	82–134	115
Tropaeolum majus	Nasturtium	1	—	65
Bryophyllum crenatum	—	1	—	12
Crassula perforata	—	1	—	0
Rochea coccinea	—	1	—	0
Sedum prealtum	—	5	—	0

with 1% ammonia solution (Coles & Waygood, 1957) did not alter the zero values given by *Sedum*. Although the addition of extracts of this species to test solutions containing, for example, *Abutilon* extract caused a drop in activity, this did not seem to be proportional to the volume of extract added and never exceeded one-third of the original activity; some inhibition was shown by a boiled *Sedum* extract.

Differences in activity among leaves of the same plant

Measurements made while a leaf in a particular position on the plant was expanding showed that (per unit of fresh weight) the aconitase activity fell in broad bean and rose in tomato (Table 3); it changed little in mustard. There was an influence of leaf position. We have the most complete information about this in mustard plants, where leaves 7, 8 and 9 may have twice the activity of the earlier ones (1–3). Some analyses of broad-bean plants grown in solution culture showed a tendency for the top leaves (7 and 8), even when fully expanded, to have two to three times the activity of the lowest (1), and the total activity per leaf to be as much as five times as great in leaves 6–8 as in the lowest.

Leaves of variegated plants

In view of the connexion between iron status and chlorophyll formation some analyses were made of green and chlorophyll-deficient areas of leaf taken from variegated plants (cf. DeKock & Morrison, 1958). Although large differences were found there was no consistent trend (Table 4). A single albino plant of broad bean showed a higher leaf aconitase than the normal plant on which it was grafted.

Measurement of fumarase activity

The same spectrophotometric method may be used to estimate fumarase (Racker, 1950), if *iso*-citrate is replaced by malate. DL-Malate was not found to be suitable for this purpose, presumably because of the inhibitory action of the D-isomer. With L-malate, activities were measured in a number of leaf extracts. Yellow fumitory (*Corydalis lutea*), a plant which accumulates fumaric acid, and *Meconopsis cambrica*, a related plant, showed higher activities (60–80 μmoles/g. fresh wt./hr.) than mustard and broad bean, for which values lay in the range 15–30. The fumarase activity seemed to be more stable than the aconitase. In *Corydalis* the activity at pH 8·5 was higher than at 8·0, but higher pH values were not tested. There seemed to be no connexion between fumarase and aconitase activities; thus *Coleus*, which yields little or no aconitase, has as much fumarase as *Abutilon*. Extracts of the yellow portions of leaves of *Sambucus*, which had an aconitase activity (111) nearly twice that of extracts of green portions (67), had rather less fumarase (37 against 51).

DISCUSSION

The main purpose of this work was to establish a reliable method for estimating aconitase activity extractable from plants of differing iron status. The method introduced by Racker (1950), although not acceptable for some purposes (cf. Herr, Sumner & Yesair, 1956), has proved very con-

Table 3. *Aconitase activity of extracts from young and older leaves*

Mature leaves were compared with leaves just unfolding or expanding on the same plant.

	Aconitase activities (μmoles/g. fresh wt./hr.)			
	Mature leaves		Younger leaves	
Species	Range	Average	Range	Average
Lycopersicum esculentum	35–80 (9)	55	27–43 (6)	33
Vicia faba	22–55 (6)	35	71–131 (6)	96

Table 4. *Aconitase activities of extracts of the leaves of variegated plants*

Some of the leaves of the *Bougainvillea* were devoid of chlorophyll and were compared with wholly green leaves. In all other cases the yellow or green areas were cut from the variegated leaves.

	Aconitase activities (μmoles/g. fresh wt./hr.)	
Species	Green tissue	Yellow tissue
Bougainvillea glabra	63, 67	21, 43, 47, 23
Coleus blumei	13, 6, 3	3, 0, 0
Hosta lancifolia	102, 96	116, 135
Remonium variegata	0	14
Sambucus nigra variegata	152, 55, 105, 90, 55, 64, 68 (average: 84)	185, 102, 84, 102, 105, 105 (average: 114)
Sidalcea rosea	35	54

venient and could if necessary be modified to measure the enzyme activity in even smaller samples of leaf than the 0·5 g. chosen here. We have not attempted to apply it to leaves too tough or fibrous to be disintegrated in a glass homogenizer; most of our results therefore refer to young and quickly growing leaves and for this reason are probably above the average that would be found for plant tissues generally.

On the other hand, the enzyme is rather labile in crude extracts [Whatley (1951–52) found none in extracts of acetone-dried powders], and so our estimates of activity must represent less than the total enzyme originally present in the leaf. However, the losses suffered in this way probably never exceeded 20–25% and the results may form a sufficient basis for the comparison of activities in leaves of various kinds.

In some plants the values obtained were considerably above the average, but suspicion attaches to some of them. For example, variegated *Abutilon* is known always to be virus-infected, and the variegated *Sambucus nigra* may also be. With higher activities towards *iso*citrate the ratio of the activity with *cis*-aconitate to that with *iso*citrate tended to be lower; this might be a sign that some enzyme other than aconitase was acting. All leaves with high aconitase activities had high ascorbic acid contents (200–400 mg./100 g. fresh wt.), but the converse was not true. The possibility cannot be altogether excluded that ascorbic acid, which has a high absorption in the ultraviolet, may sometimes interfere with the measurement of aconitase activity. The very low values for the succulents might be a reflexion of much slower growth, or they may be due to the presence of an inhibitor.

With the data accumulated it is difficult to generalize about the changes in aconitase activity as the leaf unfolds and matures. More information about these changes in *Sinapis alba* will be reported separately.

Brummond & Burris (1954) suggested that in green tissues (lupin) the enzyme was associated with a particulate fraction, and Davies (1952) showed the presence of aconitase (and fumarase) in preparations of mitochondria from pea hypocotyls. Estermann, Conn & McLaren (1959) have found it also in mitochondria from lupin cotyledons. Our own experiments do not support the idea that the enzyme is associated in any special way either with the chloroplasts or with the fractions sedimenting at 25 000 *g*. Preparations of 'mitochondria' made and tested by the procedure of Pierpoint (1959) had succinoxidase activities of the same order as (or sometimes less than) those made with our own medium modified by the addition of 0·02 M-citrate and adjustment of the pH to 7·8. However, they still carried only about 4% of the total aconitase of the extracts.

The aconitase from plant material has never been purified, but despite the earlier claims of Jacobsohn and his collaborators (cf. Jacobsohn, Soares & Tapadinhas, 1940) it seems likely that, as in animal tissues, a single enzyme catalyses the interconversions of citrate, *cis*-aconitate and *iso*citrate. There is also some evidence from the work of James (1956), and the present experiments, that the plant enzyme is sensitive to inhibitors such as $\alpha\alpha'$-dipyridyl, which might be considered to indicate that iron is involved in its action; no effect of ferrous iron has been recorded.

If one takes a figure of 25 μmoles of citrate transformed/g. fresh wt./hr. as an average value for the leaf-aconitase activity one may calculate that this corresponds to a citric acid cycle Q_{O_2} of about 10, whereas the Q_{O_2} of most leaves falls between 1 and 2 (Spector, 1956). A weakness in any such calculation is the difficulty of knowing the effective citrate concentration *in vivo*, and speculation is not very profitable. Nevertheless, the wide range of aconitase activity in the species tested suggests that in some leaves at least the enzyme cannot be a limiting factor in respiration. The changes in activity which take place as leaves mature may reflect changes in the proportion of the metabolism devoted to the synthesis of cell structures, rather than changes in the nature of the respiratory pathway, but the latter cannot be excluded.

SUMMARY

1. The spectrophotometric method of Racker (1950) for the assay of aconitase has been adapted and applied to extracts of the leaves of a variety of plants. Reasons are given for considering that the method provides a measure of the aconitase originally present in the intact leaf.

2. Results of tests on various plants are given in detail, including some obtained with the separated green and yellow parts of variegated leaves.

3. A few assays of fumarase by the same method are reported.

4. Some quantitative aspects of the values obtained are discussed.

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The Maltophosphorylase of Beer Lactobacilli

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The observation that certain beer lactobacilli grow better on maltose than on glucose (Moore & Rainbow, 1955) indicates that these bacteria metabolize maltose by a process with initial steps that do not involve the intervention of maltase. Since then, two other beer lactobacilli 'preferring' maltose for growth have been found by us. One of these (L 1), which grew rapidly and well only on maltose, of many carbohydrate substrates tested, was selected for further study as being likely to possess a restricted system of carbohydrate-metabolizing enzymes, and therefore to be uncomplicated with respect to the means by which the initial steps of maltose breakdown take place.

EXPERIMENTAL

Materials

β-Maltose. This was the monohydrate (T. Kerfoot and Co. Ltd., Vale of Bardsley, Lancs.): it was chromatographically almost free from other reducing sugars.

α-D-*Glucose* 1-*phosphate* (*dipotassium salt*). This was synthesized by a modification of Hanes' (1940) enzymic method: it was crystalline material containing less than 0·05% of inorganic P.

β-D-*Glucose* 1-*phosphate.* This was synthesized chemically as the barium salt from α-acetobromoglucose by the method of Reithel (1945) and converted into the dipotassium salt by ion exchange as described below. We are also indebted to Dr W. Z. Hassid, University of California, for a gift of a derivative of authentic *β*-glucose 1-phosphate, which was converted into the dipotassium salt for infrared examination.

Enzymes. Glucose oxidase and catalase (ex liver, 20 mg./ml.) were obtained from L. Light and Co. Ltd., Colnbrook, Bucks.

Yeast autolysate. This was prepared by incubating a suspension of pressed yeast in an equal weight of water overnight at 45°. After separating the supernatant by centrifuging, it was autoclaved at 15 lb./in.^{2} for 15 min. with 1% of filter-aid and then filtered.

Organisms. These were the strains of lactobacilli designated L3, L4, L5 and L6 by Moore & Rainbow (1955) and also strains L1 and L2. L3 and L4 are strains of *Lactobacillus brevis*, and we are grateful to Dr M. Elizabeth Sharpe for carrying out a confirmatory identification of L4. L5 and L6 are also probably strains of this species, but L1 and L2 are unidentified lactobacilli isolated from brewer's top fermentation pitching yeast by Dr D. Kulka of this Department. Stock cultures of all strains were carried as stabs in malt agar.

Media. All strains were grown on the following medium (composition/l.): yeast autolysate, 100 ml.; casein acid hydrolysate, 10 g.; *β*-maltose monohydrate, 20 g.; KH_2PO_4 (A.R.), 0·5 g.; K_2HPO_4 (A.R.), 0·5 g.; $MgSO_4,7H_2O$ (A.R.), 0·1 g.; $MnSO_4,4H_2O$ (A.R.), 5 mg.; $FeCl_3$ (A.R.), 1 mg.; KOH soln. to pH 5·0. After dispensing in suitable vessels, the medium was sterilized by autoclaving at 15 lb./in.^{2}, momentarily (for volumes less than 50 ml.) or for 15 min. (for volumes exceeding 50 ml.).

Cultural methods

Batches of cells were usually grown in two portions, each of 750 ml., contained in 1 l. conical flasks. Each portion was inoculated with 5 ml. of a culture obtained by transferring from a stock stab to a 10 ml. portion of medium in

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a test tube, which was then incubated for 2 days at 28°; the flasks were incubated at 28° for 5–7 days and shaken by hand once daily. The cells were harvested by centrifuging, combined and washed three times with sterile 0·85% NaCl soln.

Cell extracts

These were prepared from the washed cells in suspension in 8 ml. of sterile water by mechanical disintegration with Ballotini beads (0·2–0·3 mm. diam.) in a tissue disintegrator (H. Mickle, Gomshall, Surrey). Before shaking, the suspension and beads were cooled in ice, and then submitted to alternate cycles of shaking (5 min.) and cooling (5 min.) until a total time of shaking of 30–45 min. had elapsed. Cell residues and extract were then washed from the beads by successive portions of ice-cold water (50 ml. in all) and centrifuged for 20 min. at 2°. The residue, which had no relevant enzyme activity, was discarded and the supernatant frozen overnight. On thawing, a precipitate having no relevant enzyme activity was centrifuged off and the clear supernatant cell-free extract was used for enzyme experiments, usually within 2 days, although the extract retained much of its activity for months when stored at −20°. Extracts thus prepared contained about 160 μg. of total nitrogen/ml., as determined by micro-Kjeldahl.

Analytical methods

Paper chromatography. This was carried out as conventional unidimensional, descending, double runs (Jeanes, 1951) with butan-1-ol–ethanol–water (4:1:5) on Whatman no. 4 paper. Sugars were located by the aniline hydrogen phthalate reagent of Partridge (1949) and sugar phosphates by that of Hanes & Isherwood (1949).

Ion exchange. This was applied to convert barium hexose phosphate into the corresponding dipotassium salt. Columns (20 cm. × 1·2 cm.) were charged with Amberlite IR-120 resin in the H^+ form. The resin was converted into the K^+ form by treatment with aqueous 5% (w/v) KCl soln. (about 1·5 l.) until a neutral effluent was obtained. This treatment was followed by washing with water (about 2 l.) until a chloride-free effluent was obtained. An aqueous solution of barium hexose phosphate (approximately 0·5%, adjusted according to content of acid-labile phosphate) was then passed through the column at the rate of about 100 ml./hr., followed by 30 ml. of water, and the effluents were collected. The K^+ form of the resin was regenerated for further use by treatment with 5% KCl soln. until the effluent no longer gave a white precipitate with dil. H_2SO_4.

Reducing sugar. This was determined colorimetrically by the method of Schales & Schales (1945).

Esterified phosphate. This was determined as the difference between the inorganic P content of untreated samples and that of samples which had been heated for 7 min. in a boiling-water bath in the presence of N-HCl. Inorganic P was determined by Allen's (1940) method, the colour developed being measured against a reagent blank as reference solution in the EEL colorimeter (Evans Electroselenium Ltd., Harlow, Essex) with an OR-1 filter. Instrument readings were converted into quantity of inorganic P by reference to a standard curve previously constructed by submitting known concentrations of KH_2PO_4 (A.R.) to the Allen procedure.

Infrared spectra. These were carried out in a KCl disk with the Perkin–Elmer model 21 infrared spectrophotometer by Dr D. H. Whiffen and Dr K. J. Morgan of the Chemistry Department, University of Birmingham, to whom we are greatly indebted.

RESULTS

Growth of all strains on maltose. During growth on maltose medium, paper chromatography showed that, within 7 days, the maltose was almost completely removed by all strains. Simultaneously, a hexose (probably glucose), which was demonstrably absent from the inoculated culture medium, accumulated. Maltose metabolism by all strains thus appeared to involve a cleavage of the molecule to a metabolizable fragment and to glucose, which was rejected, wholly or in part, by the cells.

Further experiments to elucidate the nature of this cleavage were carried out with L 1 for reasons stated below.

Properties of strain L 1. L 1, the strain mostly used in this work, possessed unusual properties. Thus in the semi-defined CR medium of Chamberlain & Rainbow (1954) it grew well only on maltose and failed to grow on other disaccharides, hexoses, pentoses and pentose–hexose mixtures, except glucose–ribose, on which it grew relatively poorly after prolonged incubation. Best growth on maltose was obtained in CR medium adjusted initially to pH 4·8–5·0; growth took place also at more acid pH values, but not at pH values more alkaline than 5·2.

Because cells of L 1 failed to grow on glucose, it seemed unlikely that their utilization of maltose would depend on its hydrolysis to glucose by maltase. This was confirmed by incubating a cell-free extract of L 1 at 28° for 16 hr. at pH 6·8 with maltose: no glucose production was detected by paper chromatography. L 1 was therefore selected as the strain most suitable for studying the initial stages of maltose metabolism by our lactobacilli, since the situation in this case was unlikely to be complicated by the presence of maltase.

Action of extracts of L 1. Having rejected maltase activity as a factor in maltose cleavage by L 1, three other possibilities were considered: (*a*) a reaction of the type n maltose $\rightleftharpoons n$ glucose + $(\text{glucose})_n$ as described by Doudoroff, Hassid, Putman & Potter (1949) for *Escherichia coli*; (*b*) the formation of a maltose phosphate under the action of an appropriate kinase and adenosine triphosphate (ATP), followed by cleavage of this phosphate; (*c*) a reaction of the type reported by Fitting & Doudoroff (1952) for *Neisseria meningitidis*, catalysed by a maltophosphorylase:

$$\text{maltose} + (PO_4)^{3-} \rightleftharpoons \text{glucose} + \text{glucose 1-phosphate}.$$

There was no visible indication that L 1 metabolized maltose by alternative (*a*), which implies

the synthesis of polysaccharide material; this should show itself as an increased viscosity of cultures as growth proceeds. Again, in testing for (*b*), no chromatographic evidence of the formation of esterified phosphate was obtained when extracts of L 1 were incubated at pH 6·3 with 0·02 M-maltose and 0·01 M-ATP. However, esterified phosphate was demonstrated, both chromatographically and by formation of acid-labile phosphate, when mixtures containing maltose and KH_2PO_4 were incubated at pH 6·8 with extracts of L 1 (Table 1). Esterification was not increased by added Mg^{2+} ions or L-cysteine and it was not inhibited by 0·01 M-fluoride. However, it was dependent on the presence of inorganic P and maltose and it did not take place when the reaction mixture was boiled, nor when glucose was substituted for maltose in the reaction mixture. A maltophosphorylase reaction of type (*c*) was therefore indicated.

Factors affecting the activity of L 1 *enzyme.* In mixtures containing only glycylglycine (0·01 M), maltose (0·02 M), KH_2PO_4 (0·01 M) and enzyme, there was little difference in the amount of esterification at equilibrium (24 hr.) at 32° and 37°, but the amount diminished with fall in temperature down to 21°. In similar mixtures incubated at 32° for 24 hr., most esterification took place at pH values between 6 and 7. Incubation at 32° and pH 6·8 were therefore selected as suitable conditions for further enzyme studies.

When mixtures at pH 6·8 containing L 1 enzyme, 0·01 M-KH_2PO_4 and 0·01 M-maltose were incubated for 24 hr. at 32°, about 25 % of the initial inorganic P appeared as ester P, compared with about 40 % when a molar excess of maltose (0·05 M) was present (Table 1). The equilibrium of the reaction thus appeared markedly to favour maltose synthesis (see also Table 4). For the preparation of the phosphate ester (see below) it was preferred to drive the reaction in favour of the ester by using a molar excess of KH_2PO_4.

In a mixture containing KH_2PO_4 (0·01 M) and maltose (0·01 M) at pH 6·8, in which about 20 % of phosphate was esterified after incubation for 5 hr. at 32°, the amount of esterification was decreased to less than 10 % by the presence of 0·01 M-glucose (Table 1). This might be anticipated for a reversible reaction in which maltose synthesis was favoured at equilibrium.

Nature of the end products. Examination of mixtures after incubation at pH 6·8 with maltose, KH_2PO_4 and cell-free extracts of L 1 by chromatography and by electrophoresis in 0·05 M-borate buffer (pH 10) indicated that glucose and a phosphate having the mobility of α-glucose 1-phosphate were produced.

Preparation of the phosphate ester. The phosphate ester produced by the action of L 1 enzyme on maltose and inorganic phosphate was prepared as follows. The reaction mixture, consisting of KH_2PO_4 (102 g.), KOH (24·5 g.) and β-maltose monohydrate (30 g.) in 1 l. of water, was adjusted to pH 6·8 and incubated at 32° for 24 hr. with the cell-free extract derived from the cells grown in a 1 l. culture of L 1. Inorganic phosphate was then removed from the reaction mixture by precipitation as magnesium ammonium phosphate and the filtrate was concentrated *in vacuo* at 40° to about 50 ml. To this concentrate, ethanol was added, with shaking, until a slight turbidity developed. After refrigeration of the mixture overnight, the

Table 1. *Esterification of phosphate catalysed by extracts of* L 1

In Expt. A the complete reaction mixture contained (in a volume of 1 ml.): extract of cells, 0·1 ml. (16 μg. of nitrogen); KH_2PO_4, 10 μmoles; $MgCl_2$, 10 μmoles; maltose, 10 μmoles. Cysteine–HCl, NaF or glucose (each 10 μmoles) were added as shown. In Expt. B, $MgCl_2$ was omitted. Adjustment of reaction mixtures to pH 6·8 was made by addition of 10 % KOH immediately before the addition of enzyme.

		P_i (μg.) after incubation				P esterified
Incubation time (hr.)		0	3	5	22	(μg./ml./22 hr.)
Expt.	Reaction mixture					
A	Complete	62	51·5	49	47	15
	Complete, boiled	62	62	62	62	0
	No KH_2PO_4	0	0	0	0	0
	No maltose	62	62	62	62	0
	Plus glucose, no maltose	62	62	61·5	61	1
	No $MgCl_2$	62	51	49	46	16
	Plus cysteine	62	52	50	49	13
	Plus NaF	62	52·5	49	48	14
Incubation time (hr.)		0	2	5	24	(μg./ml./24 hr.)
B	Complete	62	55	49	48	14
	No maltose	62	62	62	61	1
	Plus glucose	62	61	57	56·5	5·5
	Plus extra maltose to 0·05 M	62	53	39	38	24

Table 2. *Analysis of bacterial and synthetic β-glucose 1-phosphate*

Bacterial ester	Quantity of sample analysed		Reducing sugar: Found		Reducing sugar: Purity of sample*	Inorganic P: Found		Inorganic P: Purity of sample*	$[\alpha]_D^{21}$*
	μg.	μmoles*	μg.	μmoles	(%)	μg.	μg.atoms	(%)	
(*a*) After hydrolysis	641	1·91	340	—	—	54	—	—	+8·2° (*c* 11·2)
(*b*) Before hydrolysis	641	1·91	15	—	—	2	—	—	
(*a*) − (*b*)	—	—	325	1·81	95	52	1·68	88	
Synthetic ester									
(*c*) After hydrolysis	622	1·85	285	—	—	48	—	—	+8·7° (*c* 7·5)†
(*d*) Before hydrolysis	622	1·85	15	—	—	2	—	—	
(*c*) − (*d*)	—	—	270	1·50	81	46	1·48	80	

* Calculated assuming the molecular formula of the ester as $C_6H_{11}O_9PK_2$; specific rotations are calculated assuming all impurity to be optically inactive.
† Barium salt.

Table 3. *Manometric demonstration of the presence of glucose in acid hydrolysates of bacterial ester*

Each Warburg flask contained 1 ml. of KH_2PO_4 (0·2 M, pH 5·6), 0·2 ml. of glucose oxidase (750 units/ml.), 0·1 ml. (2 mg.) of catalase and 1 ml. of substrate [glucose (1 mg./ml.), phosphate ester (2 mg./ml.) or hydrolysed phosphate ester (equivalent to 2 mg. of ester/ml.)]. Incubation was at 30°.

	O_2 absorbed (μl.)				
Time (min.)	5	10	15	20	30
Glucose	34	46	52	57	62
Phosphate ester					
(*a*) Before hydrolysis	3	3	3	3	3
(*b*) After hydrolysis	40	50	57	57	55

supernatant was decanted from the precipitated thin syrup and discarded. The syrup was dissolved in a little water and reprecipitated several times as before to remove inorganic P. This crude syrupy dipotassium salt was purified by treating its aqueous solution with a small excess of barium acetate solution and precipitating the barium hexose phosphate by careful addition of an equal volume of ethanol. The barium compound was centrifuged off, dissolved in water and the dipotassium salt was regenerated from it by ion exchange. The neutral effluent and washings were freeze-dried, the residue was taken up in water, the solution was decolorized with charcoal and filtered and freeze-dried again to yield about 50 mg. of an almost colourless solid.

A number of preparations made in this way were bulked and purified by conversion into the barium salt, which was repeatedly precipitated from aqueous solution by careful addition of ethanol (to 50 %, v/v). The dipotassium salt was then regenerated by ion exchange and the combined eluate and washings were evaporated *in vacuo* at 30–35° to about 5 ml. Absolute ethanol (5 ml.) was added dropwise to this concentrate and the supernatant was decanted from the precipitated colourless syrup. Attempts to induce crystallization of this syrup failed: it was dried to a white powder by teasing it into repeated changes of absolute ethanol, and finally dried *in vacuo* at room temperature.

Nature of the phosphate ester. The purified ester did not reduce Fehling's solution, but was readily hydrolysed by heating at 100° for 7 min. with N-HCl to inorganic P and a reducing sugar, chromatographically identical with glucose. Analysis of the preparation showed that, on hydrolysis, it yielded equimolar proportions of inorganic P and reducing sugar as glucose (Table 2). Assuming the preparation to be an anhydrous dipotassium glucose monophosphate, the analytical figures indicate it to be approx. 90 % pure.

The identity of the reducing sugar in the hydrolysate was confirmed as glucose by conventional Warburg manometric measurements of the oxygen absorbed in the presence of glucose oxidase and catalase (Bentley, 1955). Oxygen was taken up when hydrolysed, but not when unhydrolysed, ester was substrate (Table 3). With glucose, oxygen absorption was theoretical; with the hydrolysed ester, it was 85 % of theoretical, in good agreement with the results of chemical analysis.

These results, and the lability of the ester to acid, indicate that the bacterial phosphate ester is a glucose 1-monophosphate. However, two facts are indicative that the ester has the β- rather than the α-configuration: (*a*) the dipotassium salt failed to crystallize; (*b*) the previously discovered bacterial maltophosphorylase (Fitting & Doudoroff, 1952) yielded β-glucose 1-phosphate from maltose. Confirmation of the configuration was sought through its specific rotation and through its infra-red-absorption spectrum.

The specific rotations of the bacterial and synthetic esters (Table 2) are in good agreement, and the small positive rotation found is consistent

with the β-configuration ($[\alpha]_D$ of α-D-glucose 1-phosphate, dipotassium salt, is +78°).

Fig. 1 shows the close similarity between the infrared spectra of the dipotassium salts of Hassid's authentic β-glucose 1-phosphate and of bacterial β-glucose 1-phosphate. The spectra of these preparations, although lacking the strong bands at 835 and 868 cm.$^{-1}$ given by the dipotassium salt of the α-ester, have bands at 906 or 907 cm.$^{-1}$ which the α-ester lacks. According to Barker, Bourne, Stacey & Whiffen (1954), a strong band at 844 ± 8 cm.$^{-1}$ is characteristic of α-anomers of free pyranose sugars and α-glucosides, whereas the corresponding β-anomers and β-glycosides have bands at 891 ± 7 cm.$^{-1}$. Possibly the bands at 835 or 868 cm.$^{-1}$ of the α-phosphate, and that at 906 or 907 cm.$^{-1}$ of the β-phosphate, correspond respectively to those at 844 and 891 cm.$^{-1}$ reported by Barker *et al.*, the observed displacement resulting from the presence of the phosphate radical.

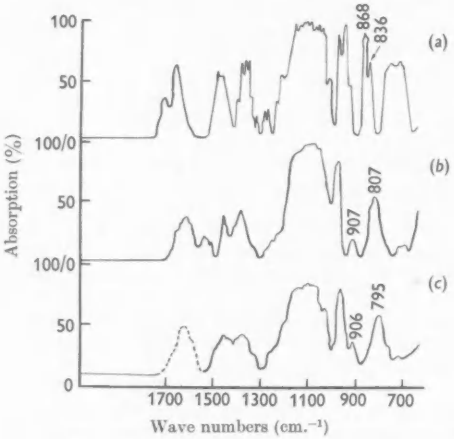

Fig. 1. Infrared spectrograms of: (*a*) α-D-glucose 1-phosphate, (*b*) β-D-glucose 1-phosphate (authentic) and (*c*) bacterial β-D-glucose 1-phosphate.

The sample of synthetic β-glucose 1-phosphate reported in Table 2 also gave an infrared spectrum similar to that of the authentic and bacterial esters: it lacked the characteristic α-band, but that at 906 cm.$^{-1}$ was less well defined, perhaps because the preparation was less pure.

These results confirm the identity of the bacterial phosphate as β-glucose 1-phosphate, with the exception that Reithel (1945) reports the $[\alpha]_D^{20}$ of his synthetic barium salt as +20·5°. The discrepancy between this value and our own cannot be ascribed to the metal ion present in the preparations studied, since the barium salt of our synthetic preparation had $[\alpha]_D^{21}$ +8·7°, which compares well with $[\alpha]_D^{20}$ +10° found by Fitting & Doudoroff (1952) for their barium salt.

Synthesis of maltose by L 1 *enzyme.* Cell-free extracts of L 1 synthesized maltose from bacterial or from synthetic β-glucose 1-phosphate in a reaction for which glucose was an essential co-substrate; 64% of the total P of the system containing equimolar initial concentrations of glucose and β-glucose 1-phosphate was released as inorganic P in 22 hr. at pH 6·8 and 32° (Table 4). The equilibrium of the system thus favours maltose synthesis. Neither maltose nor inorganic P was produced when α-D-glucose 1-phosphate was substituted for the β-anomer in this system.

Table 4. *Enzymic synthesis of maltose*

Reaction mixtures (1 ml.) contained glycylglycine buffer (10 μmoles, pH 6·8), $MgCl_2$ (3 μmoles) and enzyme (0·1 ml.; 16 μg. of nitrogen). Other ingredients were added, as indicated, in the following concentrations: D-glucose (10 μmoles); α-D-glucose 1-phosphate (dipotassium salt, 10 μmoles); bacterial β-glucose 1-phosphate (dipotassium salt, 10 μmoles). After incubation at 32°, samples were withdrawn for analysis as follows: 10 μl. for maltose (by paper chromatography) and 0·25 ml. for inorganic P. + signifies maltose clearly detected; 0 signifies maltose not detected. The total content of acid-labile P in the reaction mixtures containing α- or β-glucose 1-phosphate was 286 μg./ml.

	Formation of		
	Maltose	Inorganic P (μg./ml.)	
Time (hr.)	22	3½	22
Additions to reaction mixture			
None	0	4	8
Glucose	0	0	12
β-Glucose 1-phosphate	0	12	12
β-Glucose 1-phosphate + glucose	+	120	184
α-Glucose 1-phosphate	0	4	8
α-Glucose 1-phosphate + glucose	0	4	8

DISCUSSION

This work shows that certain beer lactobacilli contain a maltophosphorylase, like that previously found in *Neisseria meningitidis* by Fitting & Doudoroff (1952), catalysing the reversible reaction:

maltose + $[PO_4]^{3-}$ ⇌ β-glucose 1-phosphate + glucose.

The carbohydrate requirement of these lactobacilli is remarkable in that maltose is 'preferred' to glucose, which is, in fact, excreted into the

medium during growth on maltose. This phenomenon is good presumptive evidence that maltophosphorylase is responsible for the initial stages of maltose metabolism by these lactobacilli. Presumably, the β-glucose 1-phosphate produced is metabolized by the cells, but this work gives little indication of its subsequent transformation. The infrared spectra of the preparations of bacterial β-glucose 1-phosphate indicate that they are free from the α-anomer, which might be expected as a contaminant if the next step in the transformation led to the production of α-glucose 1-phosphate.

The maltophosphorylase mechanism appears to be prodigal of carbohydrate, in that a molecule of glucose is rejected for every molecule of maltose utilized. However, it carries with it the biological advantage of sparing ATP, in that a molecule of phosphorylated glucose is produced without expenditure of ATP.

It is perhaps noteworthy that the natural habitat of these lactobacilli (beer and brewer's malt wort) contains maltose as the predominant sugar, so that the maltophosphorylase system may have been elaborated by them in response to prolonged culture in the presence of maltose.

SUMMARY

1. During the growth of six strains of beer lactobacilli on maltose, glucose accumulated in the medium.

2. Cell-free extracts of L1, a strain which grew readily only on maltose, contained a maltophosphorylase, catalysing the reversible reaction:

maltose + inorganic orthophosphate ⇌ glucose + sugar phosphate.

3. The enzymically prepared sugar phosphate resembled authentic and synthetic samples of β-glucose 1-phosphate in physical properties, infrared spectrum and in its enzymic behaviour.

4. Maltophosphorylase was without phosphorolytic action on α-D-glucose 1-phosphate.

5. The preparation and some properties of β-glucose 1-phosphate (dipotassium salt) are described.

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The Cytidine Diphosphate Choline Content of Rat Brain

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The importance of cytidine nucleotides in the biosynthesis of the di-ester linkage in phospholipid molecules has been increasingly recognized in recent years. Kennedy and his co-workers have unequivocally demonstrated the reactions by which phosphatidylcholine (Kennedy & Weiss, 1956) and sphingomyelin (Sribney & Kennedy, 1958) are synthesized by the reaction of cytidine 5′-diphosphate choline with a D-α,β-diglyceride and *N*-acylsphingosine respectively. Similarly, cytidine diphosphate ethanolamine reacts with a D-α,β-diglyceride to yield phosphatidylethanolamine (Kennedy & Weiss, 1956). The biosynthesis of some other phospholipids is also dependent on cytidine derivatives [see, for example, Paulus & Kennedy (1959)]. No other nucleotide base can replace cytosine, although within the nucleotide D-ribose can be replaced by D-2-deoxyribose (Kennedy, Borkenhagen & Smith, 1959).

Studies on the cytidine nucleotide content of nervous tissues have shown that they account for only about 3% of the total nucleotides present (Mandel & Harth, 1957). Koransky (1958) in an elaborate study of rat-brain nucleotides concluded that cytidine nucleotides were absent from brain tissue; he could not isolate cytosine after total

degradation of the compounds present. In a personal communication, however, he has subsequently stated that small amounts of cytidine nucleotides are present; Miani (1956–57) has located both cytidine diphosphate choline and cytidine diphosphate ethanolamine on two-dimensional chromatograms of the total free nucleotides of brain tissue. It is apparent from the investigations of McMurray, Strickland, Berry & Rossiter (1957) that cytidine triphosphate is essential for the synthesis of cerebral lecithin via cytidine diphosphate choline, and recent work by the same group (Rossiter, Thompson & Strickland, 1960) indicates that this is also true for cerebral phosphatidylethanolamine.

In view of the pivotal position of cytidine diphosphate choline in lecithin and sphingomyelin biosynthesis it seemed of interest to determine its concentration in nervous tissue, with a method suitable for relatively small amounts of tissue. A preliminary communication has already been made (Ansell & Bayliss, 1960).

EXPERIMENTAL

Materials. Cytidine diphosphate choline, cytidine diphosphate ethanolamine, cytidine 5′-monophosphate, 2-deoxycytidine 5′-monophosphate and cytosine were purchased from Sigma Chemical Co., St Louis, Mo., U.S.A. Phosphorylcholine was prepared as the calcium salt by the method of Plimmer & Burch (1937) and freed from calcium by passing through a column of Amberlite IRC-50 resin (H^+ form). Celite 535 was purchased from Johns Manville and Co. Ltd., 20 Albert Embankment, London, S.E. 11. A generous gift of Norit-SX-Plus charcoal was made by Haller and Phillips Ltd., 14 Wool Exchange, Basinghall St., London, E.C. 2; this was boiled with N-HCl, washed first with water and then with 10% (v/v) *sec.*-octanol in ethanol (Threlfall, 1957).

[^{32}P]Orthophosphate in sterile buffered solution, containing carrier P, was purchased from The Radiochemical Centre, Amersham, Bucks.

Miscellaneous methods. Phosphorus was estimated by either the method of Fiske & Subbarow (1925) for amounts of 10–50 μg., or that of Berenblum & Chain (1938) for amounts of 1–10 μg. Phosphorus compounds on chromatograms were detected by spraying with the reagent of Hanes & Isherwood (1949), drying at room temperature and exposing to unfiltered light from a Hanovia mercury-vapour lamp. Nucleotides were located on chromatograms by reflex photography on Kodak Duostat paper; the light source was conveniently provided by a Phillips OZ 4 w lamp (unfiltered). After elution from chromatograms with dilute HCl, nucleotides could be estimated by reading the extinction at 260 and 280 mμ, and correcting for the extinction given by an eluate of a blank area.

Separation of cytidine diphosphate choline from brain tissue. Young rats (body weight 50 g.) were frozen whole in liquid N_2 and the frozen brain (5 g.) was extracted by one of the following procedures.

(*a*) The frozen tissue was homogenized in a mechanical blender with 10 vol. of ice-cold 10% trichloroacetic acid solution. After centrifuging, the extract, together with a single washing, was shaken with successive equal volumes of ether until the pH was about 4. After adjustment of the pH to 8·5–9·0 with aq. 3 N-NH_3 soln., the solution was filtered on to a column of Dowex-1 (Cl^- form) (see below). If necessary the solution could be stored at 0° at pH 7·0.

(*b*) Frozen tissue was homogenized with 10 vol. of ice-cold ethanol–water (2:1, v/v) (Kennedy & Weiss, 1956), the ethanol extract was centrifuged and then, together with washings, was reduced to about one-third of the original volume under reduced pressure at a temperature < 40°. After centrifuging, the supernatant was extracted with ether, the pH adjusted to 8·5–9·0, and the supernatant filtered as in (*a*).

The extract was slowly run through a short column of Dowex-1 (Cl^-) ion-exchange resin (50–100 mesh), followed by 30 ml. of water. Measurement of the absorption of the effluent at 260 mμ at pH 1 (the maximum for adenine nucleotides which predominate in brain) showed that at least 95% of the nucleotide material in the original extract had been retained by the resin. Elution of the resin with 100 ml. of 0·1 M-NH_4Cl–aq. 0·1 M-NH_3 soln. brought off a small amount of material absorbing at 280 mμ after acidification, and the acidified eluate was passed immediately through a short column of Norit-SX-Plus charcoal (0·3 g.) mixed with Celite 535 (1·5 g.) (cf. Threlfall, 1957) to remove nucleotides. The charcoal column was washed with water until the effluent was neutral, and the nucleotides were then eluted with 30 ml. of ethanol–water (1:1, v/v) containing aq. 0·3% NH_3 soln. After removal of solvent under reduced pressure at a temperature < 40°, the nucleotides were subjected to two-dimensional chromatography on Whatman no. 1 paper (unwashed) in ethanol–acetic acid (Kennedy, 1956), and ethanol–ammonium acetate–ethylenediaminetetra-acetic acid (EDTA) (Berger & Gimenez, 1956). The position of cytidine diphosphate choline was located with an appropriate marker and was clearly separated from a number of other nucleotides (see Results section).

Experiments with radioactive phosphorus. [^{32}P]Orthophosphate (usually 50 μc) was injected into the subarachnoid space of young rats, essentially as described by Lindberg & Ernster (1950), except that the rats were lightly anaesthetized with 2:2:2-tribromoethanol and 2-methylbutan-2-ol (Avertin, Winthrop Laboratories, New York) to facilitate the procedure. The rats recovered within 30 min. and a high incorporation of radioactive P was found in the brain nucleotide fraction after a few hours. Counting of ^{32}P was carried out in M 6 liquid counting tubes (20th Century Electronics Ltd.) with conventional ancillary equipment. Samples were assayed for radioactivity either in dilute HCl or, if the samples had been wet-ashed with perchloric acid, in 0·7 N-perchloric acid. Radioautographs of chromatograms were prepared with Kodak Blue Brand No-Screen X-ray film.

RESULTS

Identification of cytidine diphosphate choline in brain tissue

When the total nucleotides from rat brain were adsorbed on Dowex-1 (Cl^- form) and the column was eluted with 0·1 M-NH_4Cl–aq. 0·1 M-NH_3 soln.,

only a small amount of the total nucleotides were eluted. Calculation on the basis of an $\epsilon_{260\ m\mu}$ of 14 200 showed that the material eluted by 70–80 ml. amounted to only 10% of the nucleotides present in the original extract. Comparison, between an extract equivalent to 1·859 g. of tissue and an identical extract containing added synthetic cytidine diphosphate choline (0·94 μmole), indicated that this nucleotide was completely eluted with NH_4Cl–aq. NH_3 soln. The result of this experiment is shown in Fig. 1, in which the absorption of the fractions at 280 mμ (the maximum for cytidine nucleotides) is plotted against the volume of the eluate. In this particular experiment the recovery amounted to only 71% but subsequent experiments demonstrated recoveries of 80–100%. It was concluded that 70–80 ml. of 0·1 M-NH_4Cl–aq. 0·1 M-NH_3 soln. would elute all the cytidine diphosphate choline in a brain extract and separate it from 90% of the other nucleotides. Both cytidine diphosphate-ethanolamine and cytidine 5′-monophosphate were also eluted but could be separated at a later step.

After adsorption of this fraction on charcoal, elution with ammoniacal 50% ethanol and two-dimensional paper chromatography of the nucleotides in ethanol–acetic acid and ethanol–ammonium acetate–EDTA a number of substances were shown to be present both by ultraviolet photography and by radioautographs. The largest spot, to judge from the $E_{280\ m\mu}/E_{260\ m\mu}$ ratio at pH 1, seemed to be an adenine nucleotide. The position of cytidine diphosphate choline was first determined by adding synthetic material as a marker. In ethanol–acetic acid its R_F value was 0·68 and in ethanol–ammonium acetate–EDTA, 0·43. Cytidine 5′-monophosphate ran to the same position as cytidine diphosphate choline in ethanol–acetic acid but had an R_F of 0·28 in the second solvent. Cytidine diphosphate ethanolamine had an R_F of 0·57 in ethanol–acetic acid and 0·33 in the second solvent. The identity of the synthetic material with that present in the brain was further established by the following evidence:

(1) Synthetic cytidine diphosphate choline ran to the position of that present in brain in a variety of chromatographic solvents.

(2) An eluate of the spot located by means of a radioautograph was hydrolysed in N-HCl at 100° for 1 hr. (Kennedy, 1956); the hydrolysate was dried and run in one dimension in ethanol–ammonium acetate–EDTA (Berger & Gimenez, 1956). Two radioactive spots appeared in the position of cytidine 5′-monophosphate (R_F 0·26) and phosphorylcholine (R_F 0·5). The single ultraviolet-absorbing area on the chromatogram corresponded exactly with that of the radioactivity in the cytidine 5′-monophosphate position; this spot was eluted and heated with 12 N-perchloric acid at 100° for 1 hr. (Marshak & Vogel, 1951), to free nucleotide bases. This hydrolysate was diluted and freed from perchloric acid by adding KOH soln. until the neutral point was reached, and, after removal of insoluble $KClO_4$, the solution was passed over a column of Dowex-1 (Cl^-) and washed through with water. Elution of adsorbed material with 0·1 N-HCl, evaporation to dryness *in vacuo* and subsequent chromatography in propan-2-ol–HCl (Wyatt, 1951) revealed the presence of only one nucleotide base, cytosine. In an experiment in which an amount of material was separated from 145 g. of ox brain, sufficient cytidine 5′-monophosphate and phosphorylcholine were obtained on hydrolysis to permit quantitative estimation of the molar ratio of phosphorus, which was 1:1. The number of μmoles of cytidine 5′-monophosphate calculated from the absorption at 280 mμ corresponded exactly to that calculated from a phosphorus estimation.

(3) The radioactive material obtained after fractionation of a brain extract, to which had been added 0·8 μmole of synthetic cytidine diphosphate choline, was eluted from the final chromatogram, and the amounts of radioactivity and total material were determined. It was then adsorbed on to a column of Dowex-1 (Cl^-) when all the radioactivity was retained. Elution of the column with 0·1 M-NH_3 soln.–0·1 M-NH_4Cl and subsequent radioactive

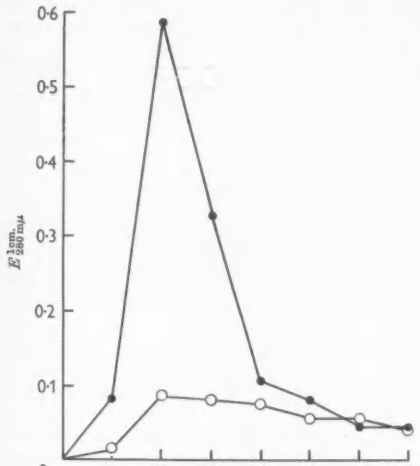

Fig. 1. Elution of brain nucleotides from Dowex-1 (Cl^-) with 0·1 M-NH_4Cl–aq. 0·1 M-NH_3 soln. ○, Brain extract (≡ 1·86 g. of tissue); ●, brain extract (≡ 1·85 g. of tissue) +0·94 μmole of cytidine diphosphate choline.

and spectrophotometric assay of 10 ml. fractions showed that the specific radioactivity was virtually unchanged (Fig. 2). Recovery of material added originally to the column both in terms of radioactivity and material absorbing at 280 mμ amounted to 89 %.

(4) It was considered that the cytidine diphosphate choline might be mixed with 2-deoxycytidine diphosphate choline, which has been found in animal tissues (Kennedy *et al.* 1959). To investigate this possibility 0·28 μmole of cytidine diphosphate choline obtained from ox brain were hydrolysed with N-HCl at 100° for 1 hr., and the hydrolysate, after being freed from HCl, was subjected to paper chromatography in ammonium acetate–sodium tetraborate–ethanol–EDTA (Reichard, 1958). Only one ultraviolet-absorbing spot was obtained corresponding in position to cytidine 5′-monophosphate (R_F 0·21). Synthetic 2-deoxycytidine 5′-monophosphate ran to a different position (R_F 0·41). A further sample (0·99 μmole) of cytidine 5′-monophosphate from the hydrolysate was tested for the presence of deoxyribose by the method of Brody (1953). Although deoxycytidylic acids react in this modified Dische reaction to give a lower colour yield than deoxyadenylic acids, the colour yield of synthetic deoxycytidine 5′-monophosphate ($\epsilon_{474\ m\mu}$ 1300) was sufficient to show that the amount of deoxy sugar in the material from brain was less than 3 %.

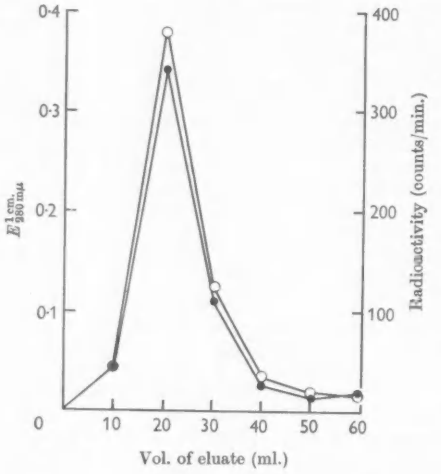

Fig. 2. Elution of radioactive brain cytidine diphosphate choline in the presence of synthetic cytidine diphosphate choline from Dowex-1 (Cl^-). Eluent, 0·1 M-NH_4Cl–aq. 0·1 M-NH_3 soln. ○, Radioactivity in counts/min.; ●, $E^{1\ cm.}_{280\ m\mu}$.

Concentration of cytidine diphosphate choline in brain tissue

Recovery of synthetic material added to brain extracts and eluted from Dowex-1 (Cl^-) was 80–100 %. Subsequent acidification, adsorption on charcoal and elution with ammoniacal 50 % ethanol did not lead to any further loss, but there was a considerable loss during the subsequent transference and chromatography. The average final recovery was 60 ± 2 % (S.E.M.), and the amount in young rat brain, calculated from recovery in seven experiments, was 0·029 ± 0·012 (S.D.) μmole/g. of brain. Additional experiments were carried out in which radioactive extracts were divided into two equal parts and carrier cytidine diphosphate choline added to one part. Calculation of the amount present from the resultant specific radioactivities of the separated cytidine diphosphate choline agreed with amounts calculated from the recovery. There was no evidence that there was any rapid post-mortem loss of the compound when extracts from the brains of animals frozen whole in liquid nitrogen were compared with extracts prepared quickly from decapitated animals.

DISCUSSION

From the investigation described in this paper it is apparent that cytidine diphosphate choline represents only a tiny fraction of the free nucleotides in rat brain and a very small proportion of the water-soluble choline derivatives. Phosphorylcholine is present at a concentration of 0·3 μmole/g. (Dawson, 1955*a*; Porcellati, 1958; Ansell & Spanner, 1959), and in addition there are measurable amounts of glycerylphosphorylcholine (Dawson, 1955*b*) and acetylcholine.

In the liver the cytidine diphosphate choline concentration is 0·1 μmole/g. (Kennedy & Weiss, 1956) [0·05 μmole/g. according to a more recent publication by Wilgram, Holoway & Kennedy (1960)]. This contrasts with 2–4 μmoles/g. for phosphorylcholine (Dawson, 1955*a*) and 0·08–0·8 μmole/g. for glycerylphosphorylcholine (Dawson, 1955*b*).

According to S. Harth & P. Mandel (unpublished work) cytidine 5′-monophosphate is the predominant cytidine compound in brain tissue. Although this material would be eluted from Dowex-1 (Cl^-) by the eluent described here, there was no evidence of any considerable concentration of the material on chromatograms. Small amounts of cytidine diphosphate ethanolamine could be detected though there was an 80 % loss of synthetic material in this procedure.

In view of the fact that sphingomyelin formation is most intense in the 2- to 4-week-old rat it might

be expected that the concentration of cytidine diphosphate choline would be higher in young than in adult animals, but no evidence for this was obtained. The phosphorylcholine concentrations in young rat brain (Ansell & Spanner, 1959) did not differ significantly from those in adult animals (Dawson, 1955*a*; Porcellati, 1958).

In view of the rapid incorporation of [^{32}P]orthophosphate into cerebral phosphorylcholine *in vivo* (Ansell & Spanner, 1959) and the steady formation of phosphatidylcholine (Ansell & Dohmen, 1957; Ansell & Spanner, 1959), there should be a very high turnover of the cytidine diphosphate choline to account for the transfer of phosphate to the D-$\alpha\beta$-diglyceride. It also seems likely that either the enzymic reaction leading to the formation of cytidine diphosphate choline or the reaction leading to the formation of the complete phosphatidylcholine molecule is affected in insulin hypoglycaemia (Ansell & Spanner, 1959) and thiopentone anaesthesia (Ansell, 1960). In any event the role of those cytidine molecules concerned with phospholipid synthesis in cerebral tissues would appear to be a central one, although turnover studies *in vivo* are difficult in view of the small amount present.

SUMMARY

1. A method for the separation of cytidine diphosphate choline from brain tissue is described.

2. Recovery of added cytidine diphosphate choline amounted to about 60% and the amount present in rat brain was calculated to be about 0·03 μmole/g. of fresh tissue.

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Techniques in Tissue Metabolism

5. CHOPPING AND SLICING TISSUE SAMPLES*

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Chopping and slicing tissue samples for metabolic work have as their objective the preparation of portions small enough to receive metabolites by diffusion, but with minimal damage to cell structure. They are therefore distinct from the blending, grinding and homogenizing which typically yield cell-free systems. Description of apparatus and procedures for chopping and slicing are relatively rare, in comparison with those for blending and homogenizing (Allfrey, 1959, includes over 40 of

* Part 4: Heald & McIlwain (1956).

these). For chopping, the only machine described is that of McIlwain & Buddle (1953) with modifications claimed by Sproull (1956) to make for ease of manufacture. Some 10 years' experience with the machine is summarized below.

Cutting animal tissues free-hand with a razor or razor blade does not yield slices as large and uniform as is desirable when they are to be manipulated during or after a metabolic experiment, and transferred individually for analysis. Deutsch & Raper (1936) described how an ordinary microscope slide, or one with a surface roughened by an abrasive, could be used to aid the cutting of tissue with a razor blade. A heavy, carefully machined plastic apparatus was introduced by Stadie & Riggs (1944) to keep the blade at a fixed distance from the tissue surface during such cutting. Experience has indicated the Stadie & Riggs cutter to be unsatisfactory in that it holds the whole of the blade rigidly parallel to the upper plate of the cutter. The tissue slice which is being cut lies between the blade and the upper plate and in cutting it is compressed by the blade against the plate (Fig. 1*B*); the part which has been cut is subject to shearing forces from the whole width of the blade as it advances in cutting. For this reason a procedure had been adopted which used a simple glass guide (McIlwain, 1951) but which was not described in detail. As it is much simpler and more versatile than the method of Stadie & Riggs (1944) and also than that subsequently reported by Majno & Bunker (1957), it is now described below.

In cutting tissue by all the methods mentioned above, the tissue or blade is normally moistened with an iso-osmotic aqueous fluid, which acts as a lubricant and minimizes distortion or 'smearing' of the tissue during the cutting. Such use of fluid is occasionally undesirable, as for example when the extracellular space of the tissue is being studied or experiments are being carried out in non-aqueous fluids. Cutting soft tissues without fluid becomes feasible if a much narrower blade is used (Rodnight & McIlwain, 1954), and it has now been found possible to use a blade only 1·2–1·5 mm. wide by means of the bow cutter illustrated in Fig. 3.

DESCRIPTION OF APPARATUS

Tissue chopper

Behaviour of original model. The machine previously described (McIlwain & Buddle, 1953; the letters below refer to Fig. 3 of that paper) has continued in use for 8 years without repair and with only minor adjustments. Current examination of its moving parts gave the following results. Arm *A*: no wear; its knife edge bearing on cam *C*1 was formed at 0·013 in. radius to avoid chipping. Arm *L* and pawl *P*: no wear. Trigger cam *C*1: very slight rounding of its knife-edge; such wear was envisaged in the original design and the cam was made 1·5 in. long and with locking screws, so that three different parts of its knife-edge could be used successively. The machine is now operating on the last of these positions: when necessary the cam can easily be removed and refaced. Cam *C*2: approx. 0·003 in. wear at the point of maximum pressure; this has not affected the thickness of slices cut by the machine. Ratchet wheel, the lead-screw and nut; shafts *Sh*1 and *Sh*2 and their bearings; the bearings of arm *A*, carriage *C* and its guides: no wear.

It is evident that the design and materials are satisfactory; the brass employed was British Standard Specification 249, the fabric Bakelite B.S.S. 972 type A, and the black Bakelite and silver steel were the best commercial qualities.

Modifications. Based on manufacturing experience (Mr H. Mickle, Mill Works, Gomshall, Surrey) and on comments from this and other laboratories (especially from Dr Ian Leslie, The Queen's University, Belfast), the following modifications have been introduced. The lead screw has been made of a larger pitch so that slices up to 0·8 mm. in thickness can be cut, and a cut-out switch has been provided so that the carriage *C* cannot over-run its free path. The part of the arm *A* which carries the blade has been made detachable so that it and the cutting table *T*, which is already removable, can be sterilized apart from the rest of the machine and easily reaffixed for cutting; this is especially valuable in preparing tissue for tissue-culture studies, and in addition Rinaldini (1959) suggests two projecting arms at the circumference of the table *T*, so that it can be rotated with minimal microbial contamination. Bearing surfaces previously made of bakelite have been replaced by light-alloy castings fitted with brass brushes, and a ball race has been fitted to the bearing surface of cam *C*2. Silver-steel rods have been replaced by rods of stainless steel, grade EN.56.AM. Many moving parts have been shielded, and the motor and the rheostat rehoused. Fig. 3 of McIlwain & Buddle (1953) still represents accurately the form and centre-to-centre distances of the moving parts and their supports, in the manufactured machines.

Further applications of the chopper to plant and animal tissues. At the suggestion of Mr N. W. Pirie, tobacco-plant leaves were found to be readily cut by the chopper with little if any bruising. Three to five leaves or portions of leaves up to 4 cm. in width were dipped in a salt solution for a subsequent experiment and put one on top of another on a filter pad, similarly dipped, on the cutting table *T*. They were held loosely in place

with a spatula and the machine started. During the cutting the solution entered the air-spaces of the leaves so that, after cutting, the strips produced were darker in colour than the original leaves, and when placed in water the strips sank while the original leaves floated.

In addition to the uses of the chopper with animal tissues which were previously described (McIlwain & Buddle, 1953) the chopper has been applied to lobes of the thyroid gland from guinea pigs (Bottari & Donovan, 1958 and personal communication) in producing uniform fragments for assay of thyrotropic hormone. It has been used to produce uniform, cell-containing suspensions of guinea-pig-lung tissue for immunological experiments (Mongar & Schild, 1960). Application to mammary gland and liver for metabolic experiments is described by Abraham & Chaikoff (1959) and by Bassham, Birt, Hems & Loening (1959). Its use in preparing tissues for culture *in vitro* is described by Leslie & Paul (1954), Paul (1959) and Rinaldini (1958, 1959), including especially the chopping of the chicken-embryo heart.

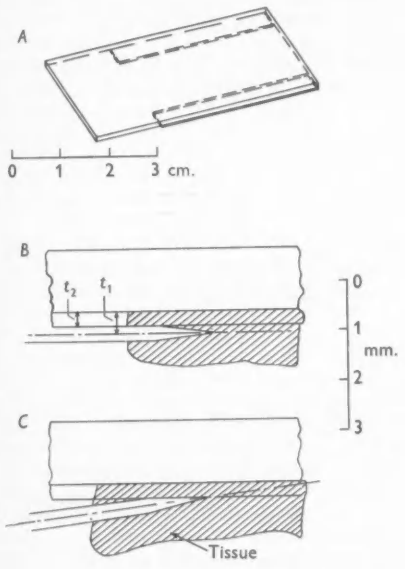

Fig. 1. Guide and blade for tissue slicing. *A*: The glass guide described in the text. *B*: Relationship between guide, blade and tissue in slicing, when the blade is held in the same plane as the guide; this is the position in the Stadie–Riggs cutter. Note that on moving forward in cutting, the slice is compressed from thickness t_1 to t_2. *C*: As *B*, with an angle of 6° between blade and guide.

Slicing tissues with blade and guide

The recessed glass guide, which has been found preferable to other apparatus, is illustrated in Fig. 1. It consists of a microscope slide, 3 in. × 1½ in., with ridges on its long edges which leave the centre of the slide as a recess. It is normally used in conjunction with a strip of razor blading 118 mm. × 18 mm., obtainable as a stage in the manufacture of the type of safety razor blades which have one cutting edge (Valet 3-blade strip, Gillette Industries Ltd., Syon Lane, Osterley, Middlesex). There is a particular manoeuvre in the use of the blade and guide which minimizes the rubbing of the blade on the tissue being cut; it is as follows.

The tissue is placed on a heavy table which raises it about 7 cm. above the bench (Fig. 2). The guide and blade are dipped in the fluid medium to be used

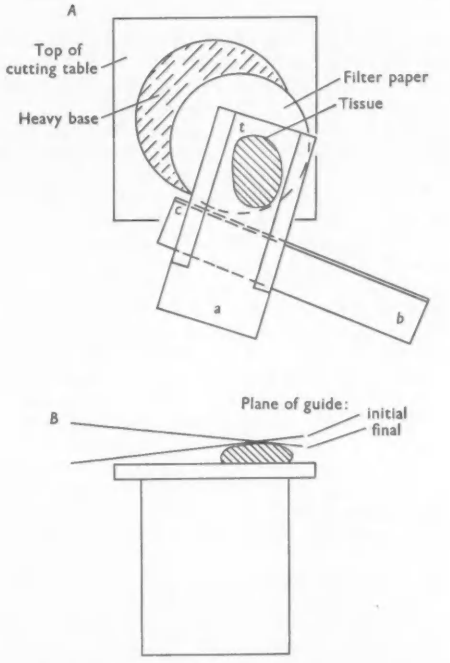

Fig. 2. Slicing tissue with blade and guide. *A*: In plan; the tissue lies on a circle of moist filter paper on a cutting table. The guide is held at *a* between the left thumb and forefinger, and the blade at *b* with the right thumb and fingers; *t*, initial position of the corner *c* of the blade when sweeping out air bubbles (see text). *B*: Elevation of cutting table and tissue showing the change in the plane of the guide during cutting, as seen from the left-hand side of *A*.

in a subsequent experiment, and the excess of medium is removed by shaking. The guide is held nearly horizontally with its ridges below, and the blade brought up from underneath it so that the two ridges of the guide touch the blade, but only at points near its cutting edge; the plane of the blade is a few degrees different from that of the guide (Fig. 1*C*; see below). The pressure of guide on blade is only a little greater than the weight of the guide, which is 8–9 g. Maintaining this relative position of blade and guide, both are lowered on to the tissue as shown in Fig. 2*A*. They rest lightly on the tissue, with a pressure about equivalent to the weight of the guide. The blade is then advanced into the tissue, cutting it with the usual combination of forward and side-to-side movements. As this is done the plane of the guide is altered as shown in Fig. 2*B*, while still keeping about the same angle between blade and guide. Thus at first the 'handle' end of the guide is lowest, while at the end of the cut it is raised above the rest of the guide. After cutting, the tissue is transferred with the blade or the guide (to whichever it is adhering) into a shallow dish of medium, and by jerking the blade or guide the slice is floated free.

The procedure described should be found satisfactory in cutting slices from a tissue surface which is slightly convex; but with a tissue surface which is flat or slightly concave, air bubbles are likely to be trapped when the guide is first placed on the tissue, and to cause holes in the slices which are cut. In such cases, the blade and guide are first applied to the tissue while the tip *c* of the blade is in the position indicated in Fig. 2*A*, and the blade drawn towards the operator until it occupies the normal starting position. Its movement sweeps out air which would otherwise be trapped, and brings the guide into complete contact with the tissue. It can readily be seen through the transparent glass guide whether this has been done; the surface of the guide should not be ground.

Preparation of the recessed glass guide. Glass microscope slides 76 mm. × 38 mm. (3 in. × 1·5 in.) and 1–1·2 mm. thick, with their edges ground smooth, and also no. 0 or no. 1 coverslips, 64 mm. × 44 mm. (2·5 in. × 1·75 in.) are put one by one into chromic acid–sulphuric acid cleaning mixture and after some hours washed well and dried in an oven at 105°. The coverslips are placed on a flat clean glass surface [Opalite: James Clark and Eaton Ltd., London, S.E. 1] and cut to strips 5–6 mm. × 64 mm., with a steel rule and a diamond. Between each cutting the Opalite and the rule must be wiped free of fine glass slivers with a soft paper tissue or cloth.

A Canada balsam–xylene solution (approx. 2:1, v/v) is prepared and with a glass rod four small spots of the solution are placed at each edge of a slide in the positions to be occupied by the strips of coverslip (Fig. 1*A*). A strip is taken in forceps and lowered on to each set of balsam spots. These should flow to a complete film between slide and strip, with a little excess of fluid appearing at the edges. Further spots of balsam–xylene are placed on the affixed strips, and further strips placed above them. The spots of fluid are transferred with a rod already drained from balsam and are not added as drops. The strips of coverslip are aligned with each other and with the edge of the slide, and the slide carrying them is put horizontally in an oven at 105° overnight. Excess of balsam is then wiped off with a tissue moist with xylene. If the balsam has retracted and left a gap under a strip of coverslip, the gap is filled with xylene–balsam solution. The slides are left for a further day at 105°. Measurement has shown the thickness of their ridges to be stable for a year or more after this treatment.

The guides are conveniently made in batches from six to twelve, and are then calibrated with an engineer's micrometer. Measurements are made to 0·005 mm., of the thickness of the slide at its centre, and of the thickness of the slide plus coverslips at three points on each ridge. The difference between measurements at the centre and at the ridge gives the thickness of the ridge and is the main factor conditioning the thickness of slices cut with the guide. The six measurements of thickness of ridge should agree to within 0·01–0·02 mm. in a satisfactory guide. No. 0 coverslips have been found about 0·10 mm. in thickness, and no. 1, 0·125 mm. Guides (*a*) made from two no. 0 coverslips have averaged 0·24 mm. in depth; those (*b*) from one no. 0 and one no. 1, 0·265 mm.; and those (*c*) from two no. 1 coverslips, 0·285 mm.

Thickness of slices cut. The thickness of slices cut with the guide and blade can be judged by Fig. 1*B* and *C*, to be conditioned by the depth of the recess in the guide and by the thickness of the blade. However, it is less than the sum of the depth and half the thickness of the blade by an amount which depends on the angle between the blade and guide. This, and the pressure exerted by the guide on the tissue, are individually-conditioned matters which should be standardized as far as possible by an investigator, who should then measure the thickness of the slices which he cuts. The following measurements were made according to Warburg (1923) with slices of guinea-pig cerebral cortex, specific gravity 1·03, cut with the blades of thickness 0·26 mm. in the fashion recommended and with guides (*b*) and (*c*) above. Immediately after the slices were cut, they were washed from the guide, drained from fluid medium and were weighed on a torsion balance to the nearest mg.; they were then floated in a Petri dish of phosphate–

glucose saline (McIlwain, 1951) above paper ruled in millimetre squares. The data showed that with guide (*b*), slices were 0·32 mm. and with guide (*c*), 0·34 mm. in thickness. By keeping the blade parallel to the guide, slices were about 0·06 mm. thicker.

Bow cutter. The bow cutter was designed to enable soft tissues to be cut without aqueous fluid; this depends on its having a narrow blade kept rigid by tension (Fig. 3). The narrow blades are obtained, from the strips of razor blading described above, by one of the following methods. (i) The cutting edge of the blade is gripped in the centre of the carefully cleaned jaws of a sheet-metal bending machine (an Edwards swing-beam sheet-metal folder). The machine is operated as though a right-angle bend were to be made in the blade; as the blade is hard and brittle, it breaks much before this degree of bending has been achieved, and in a proportion of cases gives the narrow blade desired. The yield of successful blades with a new bending machine was about 50% of those bent, but slight irregularities in the jaws reduce this yield; a piece 7 cm. or more in length is required. (ii) A strip of the blade 1·2 mm. wide including the cutting edge is gripped between metal bars in a vice. A strip of copper sheeting about 15·4 cm. × 3·75 cm. (6 in. × 1·5 in.) is bevelled at one of its long edges, and this edge used with emery to cut through the blade by abrasion. The narrow blade when released from the bars is intact and 1·2–1·5 mm. wide.

The frame of the bow cutter (Fig. 3) is made from Stubbs's silver-steel wire, $\frac{1}{16}$ in. (1·6 mm.) in diameter. A length of about 22 cm. is bent to a shape which will become that shown in Fig. 3 when compressed (see below). It is tinned with solder at its two ends. The narrow strip of blading, about 7 cm. long, is also tinned at its ends. The frame is fixed between nails on a block of wood so that its ends are under compression of rather more than 500 g., but not under torsion. The blade and frame are brought together and soldered. The frame is released when cold, and the blade should then be quite flat (not twisted) and require a weight of about 500 g. applied to its ends to cause it to bend.

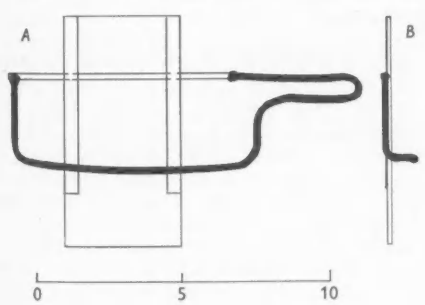

Fig. 3. The bow cutter. *A*, Plan; *B*, end elevation, showing its position in relation to a glass guide when both are used in slicing tissue.

The tension on the narrow blade in the bow cutter is approx. 20 kg./cm.^{2} and keeps the blade sufficiently rigid for it to be handled in much the same way as one of the wide blades from which it was made. In particular it can be used with the recessed glass slide to give slices of defined thickness from soft tissues without the use of saline. Metabolic experiments with slices prepared in this way from cerebral cortex, liver and kidney have been described by Rodnight & McIlwain (1954). The bow cutter weighs 3·5–4 g., and the original blades 4·2 g.

DISCUSSION

Comment may be made on the choice of chopping or slicing methods in the preparation of tissues, for their relative merits are not always understood. The tissue chopper was designed to give cell-containing preparations from small and irregular specimens, obtained for example at biopsy or from small organs or small parts of organs. It remains valuable in this and other situations in which a cell-containing preparation is required from the whole of a tissue sample, without leaving a residue and thus introducing the likelihood that the material used is not representative of the whole sample. For these purposes the tissue is chopped in one plane only, to a series of slices. The chopper has also been found valuable in sampling heterogeneous tissues, for after chopping in two planes to a series of prisms, the tissue can be suspended in a fluid, mixed and representative samples taken for each of a number of vessels. Also, when finely subdivided, it can be suspended and dispensed by pipette though remaining a cell-containing tissue. The chopper has a further use in cutting, mechanically, larger specimens of such a size that slicing by hand would be tedious.

Slicing remains the most suitable process when a moderate amount of relatively uniform tissue is to be prepared. Slicing can yield relatively large sheets of tissue, larger than those available by chopping, and thus with a proportionally smaller degree of damage. Also, it can yield up to about 150 mg. of tissue as a single sheet thin enough to meet the requirements of metabolic experiments. It is advantageous to have the tissue as a single sheet when it is to be transferred and analysed.

Slices of these dimensions cannot be cut free-hand but are readily prepared with blade and guide as described above. After this method of

slicing had been devised and used for some years, Majno & Bunker (1957) described a more elaborate apparatus for slicing in which a blade is fixed horizontally on a stand and the tissue moved by hand across and above the blade. This apparatus also yields such slices. Two models of the apparatus have been examined, one with the mounted microtome blade described by Majno & Bunker (1957) and another employing a strip of razor blading. With both, the cutting process could not be watched; the slice was more difficult to transfer, and an aqueous medium was always required in cutting. [*Added August* (1960). In the apparatus of Hultin, Arrhenius, Löw & Magee (1960), also, the slice is not visible during cutting and is again subject to shearing forces from the whole width of a cutting blade.] The guide described here allows the progress of the cutting to be seen and thus to be better controlled; with the bow cutter it allows slicing without added aqueous fluids. For these reasons the simple recessed guide used as described above appears the more satisfactory and versatile instrument.

SUMMARY

1. The tissue chopper previously described has undergone development and has been further applied.

2. For slicing tissues, the preparation and use of a simple guide is described.

3. A bow cutter is described for use with the guide when cutting tissue in the absence of added aqueous fluid.

I acknowledge with gratitude help and information from Mr H. L. Buddle and Mr H. Mickle regarding the tissue chopper; from Mr A. McNeil regarding the cutting guide and from Mr H. L. Buddle and Dr R. Rodnight regarding the bow cutter.

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Use of Carbon Monoxide for Detecting Denaturation in Haemoproteins

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Through the work of Hoppe-Seyler (1889) it became known that haemoglobin forms a compound with carbon monoxide. Hill (1926, 1930) observed that combination with carbon monoxide takes place also with the isolated prosthetic group of haemoglobin in the form either of haem or of its pyridine compound pyridine haemochromogen. The reactivity towards carbon monoxide observed both with haemoglobin and its prosthetic group is by no means characteristic of haem compounds in general. On the contrary, many haem compounds, among them the important class of intracellular respiratory pigments known as cytochromes, appear as a rule to be inert towards carbon monoxide. Keilin, in his pioneer investigations on these respiratory pigments, encountered only one exception to this rule in animal tissues, namely cytochrome a_3 (Keilin & Hartree, 1939).

As soon, however, as their native structure is disorganized by exposure to alkali, or by other influences, cytochromes as a rule can become reactive towards carbon monoxide. It thus appears that the reaction with carbon monoxide in the neutral pH range provides a convenient means of detecting denaturation. In numerous publications, however, investigators failed to report the behaviour of their preparations towards carbon monoxide, leaving it open to doubt whether their

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observations were made with native cytochromes, or with partly or totally denatured compounds. This is the more surprising since the test had already been in use for years (see, for example, Slater, 1949, 1958; Strittmatter & Ball, 1952).

Though numerous investigations involving carbon monoxide–haem compounds, beginning with the observations of Hoppe-Seyler and including the recent studies of Smith (1959), have contributed much to our knowledge of haem–carbon monoxide compounds, we are unaware of any systematic survey of the use of carbon monoxide for detecting denaturation of haemoproteins. Correlation of data reported in the literature is not easy. Earlier experiments were carried out with the help of gasometric methods or by visual microspectroscopy, with the result that the spectral changes in the ultraviolet and the near-ultraviolet range of the spectrum were often neglected.

Moreover, spectroscopic observations were made under insufficiently defined conditions, as it was not at first realized that haem compounds are susceptible to oxidative degradation in a sodium carbonate medium and that they are strongly affected by changes in pH, the nature of the solvent and the degree of dispersion of the pigment (Keilin, 1926; Keilin, 1943, 1949; Shack & Clark, 1947; Smith, 1959).

It was considered desirable to repeat earlier experiments by means of a quartz spectrophotometer under better defined and comparable conditions. The spectra changes occurring in a carbon monoxide test are illustrated on a simplified model of a denatured haemoprotein. An example of the application of the test to a haemoprotein preparation isolated from heart muscle will be described.

MATERIALS AND METHODS

Haemin. The method of Labbe & Nishida (1957) was used for purification of haemin from ox blood.

Haemoglobin. A sample prepared from ox erythrocytes was provided by Dr D. H. Deul in this laboratory. The preparation contained much methaemoglobin.

Cytochrome c. A preparation from horse heart isolated by the method of Keilin & Hartree (1945) was used.

Carbon monoxide. CO was generated by dropping pure formic acid into warm conc. H_2SO_4. The gas was collected in a gasometer above concentrated aqueous $(NH_4)_2SO_4$.

Spectroscopy. For preliminary qualitative and semi-quantitative spectroscopic examinations, a Zeiss–Winkel microspectroscope was used.

Absorption measurements were carried out with a Zeiss spectrophotometer (PMQ-II) with absorption cells with a light path of 1 cm. For low extinctions 2 cm. cells were used and the values recalculated for a light path of 1 cm. Readings were taken at 1 mμ intervals in the critical parts of the spectrum and at 5 or 10 mμ intervals elsewhere. The wavelength-scale calibration of the spectrophotometer was checked against two emission bands of the hydrogen lamp.

Procedure for the carbon monoxide test. To 5 ml. of the solution containing the haem compound a few grains of $Na_2S_2O_4$ were added. The sample, contained in a wide test tube, was saturated with CO by passing through a finely divided stream of the gas for 5 min. The sample was then transferred to an absorption cell and a few additional grains of $Na_2S_2O_4$ were stirred in to bring the final concentration to about 1%. To prevent reoxidation and escape of CO, the sample was covered with a layer of liquid paraffin, about 4 mm. high, and the absorption cell closed with a rubber lid. The complete spectrum was measured within 10 min. The initial absorption values were unchanged after 30 min.

Difference spectra. Bubbling CO through the solutions usually increased the background absorption of the samples to a variable extent by changing the state of dispersion of the pigments. For the sake of uniformity the difference spectra were calculated on the assumption that at 540 mμ the extinction of the reduced and the CO-reduced compounds were equal.

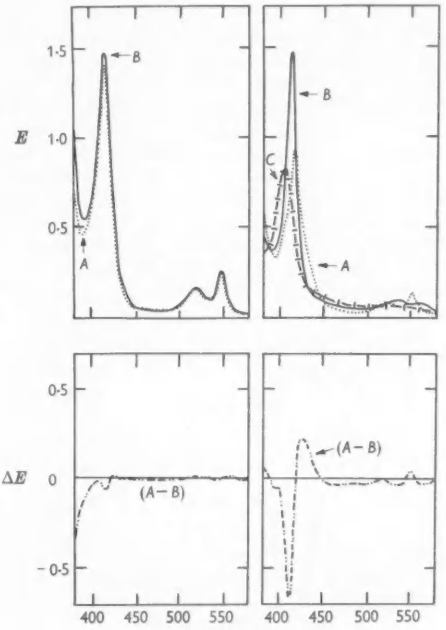

Fig. 1 Fig. 2

Fig. 1. Effect of CO on the spectrum of native cytochrome *c*. 9·16 μM-Cytochrome *c* in 0·05 M-phosphate, pH 7·4. *A* ···, $+Na_2S_2O_4$; *B*, —, $+Na_2S_2O_4+CO$; —·—, difference spectrum $(A-B)$, adjusted so that $\Delta E_{540\ m\mu}$ is 0.

Fig. 2. Effect of CO on the spectrum of denatured cytochrome *c*. 9·16 μM-Cytochrome *c* in 0·05 M-phosphate, pH 7·4. *A*, ···, $+Na_2S_2O_4$; *B*, —, $+Na_2S_2O_4+CO$; —··—, difference spectrum $(A-B)$, adjusted so that $\Delta_{540\ m\mu}=0$; *C*, −|−|, oxidized with $K_3Fe(CN)_6$.

RESULTS

Fig. 1 shows the absorption spectrum of a cytochrome *c* preparation, Fig. 2 the spectrum of the same preparation after extensive denaturation by treatment with 0·6 N-KOH for 24 hr. at 32° and subsequent neutralization. With the extensively denatured preparation the α- and β-bands at 550 and 520 mμ are flattened by treatment with CO and replaced by rather diffuse bands at 562 and 533 mμ respectively, whereas the Soret band is sharpened and shifted from 416 to 412 mμ. The slight increase in the Soret band brought about by CO (Fig. 1) is no doubt due to contamination with denatured cytochrome *c*, which is usually present in small amounts in cytochrome *c* prepared by the above-mentioned procedure. Fig. 2 includes the spectrum of the same denatured cytochrome *c* preparation after treatment with $K_3Fe(CN)_6$. There is no indication that the CO compound of the reduced cytochrome *c* was contaminated by oxidized cytochrome *c*. However, the resemblances between the flattened α- and β-bands of the oxidized form and the CO compound of the reduced pigment make it advisable to be sure of complete reduction by adding excess of $Na_2S_2O_4$.

Haemoglobin was denatured by treatment with alkali until it had lost its capacity to combine with O_2. The spectrum (curve *A*, Fig. 3) showed the typical sharp α- and β-bands of a haemochromogen. Addition of CO (curve *B*, Fig. 3) caused a considerable weakening and flattening of these bands, whereas the Soret band was increased in intensity. Curve *C*, Fig. 3, shows for comparison the spectrum of the CO compound of native haemoglobin. This is similar to that of the CO compound of denatured haemoglobin, but the absorption is greater.

In the next set of experiments an artificial protein-free haemochromogen served as a simple model of a denatured haemoprotein. The denatured

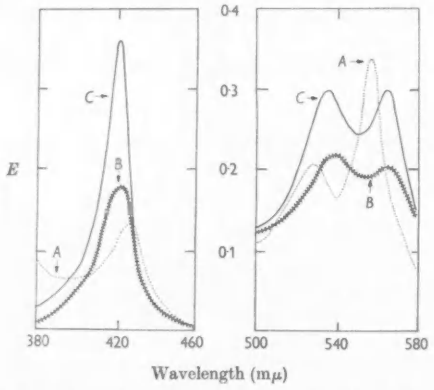

Fig. 3. Effect of CO on the spectrum of native and denatured haemoglobin. Haemoglobin was denatured by treatment with 0·2 N-NaOH for 1·5 hr. at 20°. The spectrum was measured in 0·1 M-phosphate, pH 7·4, with 20 μM-haemoglobin in visible range and 4 μM in u.v. range. *A*, ..., denatured haemoglobin + $Na_2S_2O_4$; *B*, –|–|, denatured haemoglobin + $Na_2S_2O_4$ + CO; *C*, —, native haemoglobin + CO.

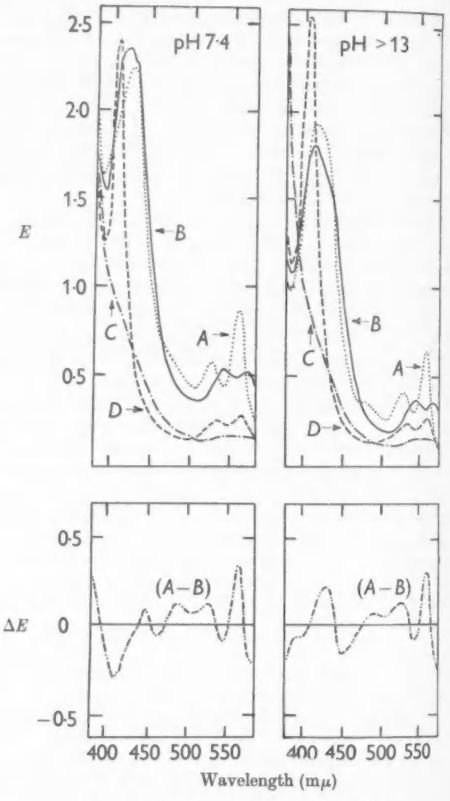

Fig. 4 Fig. 5

Fig. 4. Effect of CO, at neutral pH, on spectrum of pyridine haemochromogen in the presence of a low concentration of pyridine. All solutions contained 2 mM-haem, 0·1 M-phosphate, pH 7·4, and 1% of $Na_2S_2O_4$. *A*, ···, +0·075% (w/v) of pyridine; *B*, —, 0·075% (w/v) of pyridine + CO; *C*, —·—, no addition; *D*, - - -, +CO; —· ·—, difference spectrum (*A* – *B*).

Fig. 5. Effect of CO, at high pH, on spectrum of pyridine haemochromogen in the presence of a low concentration of pyridine. 0·1 M-Phosphate buffer, pH 7·4, was replaced by 0·1 N-NaOH. Other conditions and *A*, *B*, *C* and *D* were as in Fig. 4.

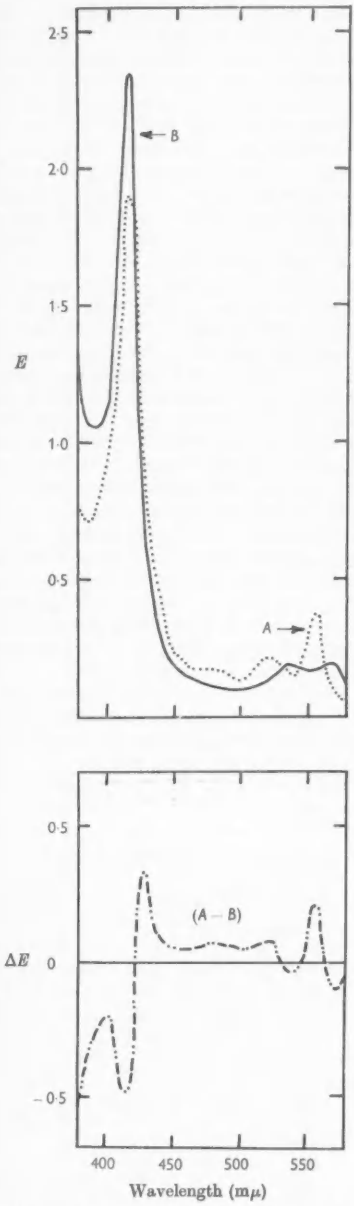

Fig. 6. Effect of CO on spectrum of pyridine haemochromogen in the presence of a high concentration of pyridine. Both solutions contained 1 mM-haem, 0·1 N-NaOH, 20% (w/v) of pyridine and 1% of $Na_2S_2O_4$. *A*, · · ·, no further addition; *B*, —, +CO; —· · ·—, difference spectrum (*A* − *B*).

protein linked to the haem iron was replaced by pyridine. The CO test was carried out under two sets of conditions. In the first, the pyridine concentration was kept comparatively low, in order to obtain a maximum effect with CO, by favouring the right-hand side of the equilibrium equation

$$\text{pyr–Hm–pyr} + \text{CO} \rightleftharpoons \text{pyr–Hm–CO} + \text{pyr},$$

where pyridine is represented by pyr and haem by Hm, the hyphens denoting attachment to the haem iron. Figs. 4 and 5 show the effect of CO at nearly neutral and at strongly alkaline pH, respectively. In the second set of experiments a high pyridine concentration (20%) corresponding to the standard conditions for preparing pyridine haemochromogens was used (cf. Hartree, 1955). Even here a marked change of the spectrum resulted (Fig. 6). A flattening effect of CO on the α- and β-bands is evident in all three cases, irrespective of the variation in pH and pyridine concentration. The Soret bands behave differently. With 20% of pyridine there is an increase in absorption with no appreciable shift in the position of the band (Fig. 6). With the low pyridine concentration less

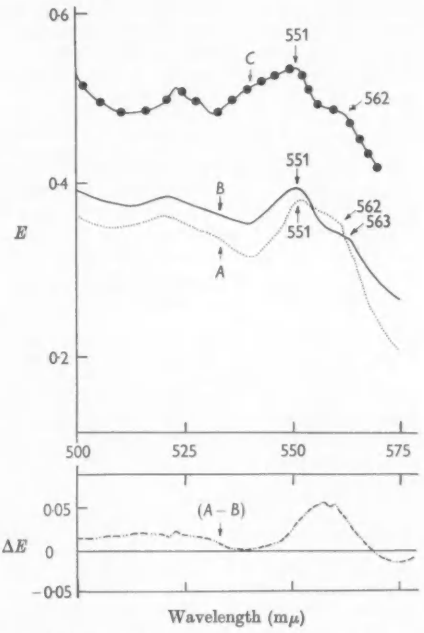

Fig. 7. Effect of CO on spectrum of a cytochrome *b* preparation. The preparation was solubilized with 1% of deoxycholate in 0·1 M-phosphate buffer, pH 7·4. *A*, · · ·, +$Na_2S_2O_4$; *B*, —, +$Na_2S_2O_4$+CO; *C*, —•—, +sodium succinate; —· · ·—, difference spectrum (*A* − *B*).

sharp Soret bands are obtained; the shapes of the long-wavelength slopes suggest the presence of other components (Figs. 4, 5). Yet whereas at strongly alkaline pH a small decrease in absorption takes place, at pH 7·4 the Soret band slightly increases. For comparison the spectra of reduced haem and haem–CO in the absence of pyridine are also shown in Figs. 4 and 5.

Fig. 7 illustrates an application of the CO test in investigations concerning cytochrome *b*. In an attempt at purifying this cytochrome from ox heart according to a fractionation procedure of Bernstein & Wainio (1958), a preparation was obtained, which upon addition of succinate showed two weak absorption bands at 551 and 562 mμ, respectively. Upon addition of $Na_2S_2O_4$ a broad and strong absorption band appeared in the range 551–560 mμ. The difference in the spectra obtained with succinate and $Na_2S_2O_4$, respectively, appears to correspond to the amount of denatured haemoprotein present. The preparation which we obtained probably contained both cytochrome c_1 and cytochrome *b* with most of the cytochrome *b* in an enzymically inactive, denatured state. Treatment in the presence of $Na_2S_2O_4$ with CO resulted in a spectrum that showed a striking similarity to that obtained with succinate. The difference spectrum of Fig. 7 exhibits a peak at 558–560 mμ, corresponding presumably to the α-band of denatured cytochrome *b*.

DISCUSSION

Table 1 summarizes the main spectral changes in our experiments. Wherever carbon monoxide compounds were formed with denatured haemochromogens or with pyridine haemochromogens, two kinds of changes in the absorption bands took place. (i) A shift of the position of the bands towards the longer wavelengths was generally observed with α- and β-bands; a shift towards the shorter wavelengths was observed with the γ-bands, except in the experiment involving the presence of 20% of pyridine, in which no shift was found with the γ-band. (ii) In most cases there occurred also an appreciable change in the height of the bands. The bands in the visible part of the spectrum decreased, whereas the γ-bands usually increased in height and sharpness. In Table 1 an increase or decrease in a band is indicated merely as such without giving numerical values; such values could bear full significance only if the extent of denaturation of the haemoprotein had been estimated by a different method and could be quantitatively correlated. The absorption spectra and the difference spectra in Figs. 1–7 nevertheless give a measure of the flattening or sharpening of the bands due to reaction with carbon monoxide. In the experiments in which pyridine haemochromogen served as a model of a denatured haemoprotein, some additional minor peaks or shoulders were observed which are not included in Table 1, e.g. the shoulder at 430 mμ of the pyridine haemochromogen–CO compound depicted in Fig. 4, which no doubt indicates residual pyridine haemochromogen. The appearance of these shoulders is in keeping with the observations of other investigators. When Smith (1959) attempted to prepare pyr–Hm–CO by applying carbon monoxide to pyr–Hm–pyr, residual unconverted pyr–Hm–pyr invariably persisted. He was, however, able to prepare the pyr–Hm–CO compound in full yield by a different sequence of reactions, i.e. by preparing first

Table 1. *Effect of combination with carbon monoxide on absorption bands of haem compounds*

		Absorption bands in reduced state						Change in extinction	
		Before CO treatment			After CO treatment				
	pH	α	β	γ	α	β	γ	Visible bands†	Soret band†
Cytochrome *c* (native)	7·4	550	520	415	550	520	415	−	+
Cytochrome *c* (denatured)	7·4	550	520	416	562	532	412	−	+
Pyridine haemochromogen (0·075% of pyridine)	7·4	560	527	426	572	540	416	−	+
Pyridine haemochromogen (0·075% of pyridine)	13	560	526	416	570	542	412	−	+
Pyridine haemochromogen (20% of pyridine)	13	555	523	418	568	538	418	−	+
Haemoglobin (native)	7·4	555	.	430	566	538	420	.	.
Haemoglobin (denatured)	7·4	560	.	425	568	540	422	−	+
Cytochrome *b* preparation	7·4	558–563	.	428*	563‡ (551)	.	419*	−	.
Haem	7·4	540–580	.	.	564	532	407	.	.
Haem	13	540–580	.	.	564	532	406	.	.

* Value obtained with a different cytochrome *b* preparation.
† +, Increase in *E*; −, decrease in *E*.
‡ Shoulder.

CO–Hm–H_2O and then adding pyridine. In our experiments we did not attempt to achieve a 100% conversion into the carbon monoxide compound, as it was our main purpose to imitate as closely as possible, on a simpler model, the procedure applied in the test for denatured haemoproteins. The measurements were also deliberately restricted to a spectral range where dithionite does not interfere with the spectrum and therefore can conveniently serve as a reducing agent. In the spectral range below 390 mμ rather elaborate methods of reduction have to be resorted to, which do not commend themselves for a simple routine test.

The experiment illustrated in Fig. 7 shows the use of both an enzymic method and the carbon monoxide test for estimating the degree of denaturation in a cytochrome *b* preparation. Disregarding the roughly constant difference in extinctions of curves *B* and *C*, which may be attributed to a change in background absorption (compare 'Difference spectra' in the Materials and Methods section), the agreement of the two methods appears reasonable. A comparatively simple enzymic test was possible in this particular case, owing to the fact that the crude cytochrome *b* preparation still contained some succinic dehydrogenase. Further purification of the cytochrome *b* would be most likely to lead to a preparation no longer reducible by succinate. At such a stage, failure of the absorption bands to appear on addition of succinate would no longer be conclusive. It might be due either to absence of succinic dehydrogenase or to a total denaturation of the cytochrome *b*. An enzymic method gives the most direct evidence of the native state of a cytochrome and, wherever practicable, deserves preference. The carbon monoxide test, on the other hand, has the advantage that it can also be applied when conditions for an enzymic test are not known or where they cannot easily be achieved.

The effect of combination with carbon monoxide on the spectra of haem and of pyridine haemochromogen, contained in an aqueous medium, appears to be connected with, if not due to, a depolymerization of the previously dimeric haem compound (Shack & Clark, 1947; Smith, 1959). It is a subject for further investigation to show if one could account in an analogous way for the spectral changes observed upon addition of carbon monoxide to denatured haemoprotein.

SUMMARY

1. The effects of carbon monoxide on the spectra of some native and denatured haem compounds are compared.

2. Pyridine haemochromogen was used as a model of a denatured haemoprotein reacting with carbon monoxide.

3. The usefulness of carbon monoxide as a method of detecting denaturation of haem proteins is discussed.

I wish to thank Professor E. C. Slater for his interest and advice. The skilful assistance of Mr H. de Klerk is also gratefully acknowledged.

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